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Distribution et déterminisme de l'abondance, de la diversité et de la structure des communautés microbiennes à différentes échelles spatiales pour l'appui au développement agricole

Nicolas Chemidlin Prévost-Bouré

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Habilitation à Diriger les recherches
Présentées devant
l'Université de Bourgogne Franche-Comté

Date de soutenance : le 04-07-2018

Distribution et déterminisme de l'abondance, de
la diversité et de la structure des communautés
microbiennes à différentes échelles spatiales pour
l'appui au développement agricole

Par

Nicolas CHEMIDLIN PRÉVOST-BOURÉ

Maître de Conférences à AgroSup Dijon

Jury

Loic Bollache, Professeur Université de Bourgogne Franche-Comté, Dijon [Rapporteur]

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Jean-Luc Chotte, Directeur de Recherches IRD, Montpellier [Rapporteur]

Dominique Arrouays, Ingénieur de Recherche INRA, Orléans [Examineur]

Isabelle Feix, Experte Nationale Sols ADEME, Angers [Examineur]

Dirk Redecker, Professeur Université de Bourgogne Franche-Comté, Dijon [Examineur]

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SOMMAIRE

CHAPITRE 1 – PARCOURS SCIENTIFIQUE	1
1 HISTORIQUE ET POSITIONNEMENT DANS L’EQUIPE DE RECHERCHE	2
2 CV	4
CHAPITRE 2 – SYNTHÈSE DES ACTIVITÉS DE RECHERCHE	21
1 INTRODUCTION	22
2 THÉMATIQUE, OBJECTIFS ET STRATÉGIE DE RECHERCHE	25
2.1 THÉMATIQUE DE RECHERCHE ET OBJECTIFS	25
2.2 STRATÉGIE DE RECHERCHE	26
3 ÉTUDE DES ÉMISSIONS DE CARBONE PAR LES SOLS EN FORÊT TEMPÉRÉE DÉCIDUÉE	27
4 DISTRIBUTION SPATIALE ET DÉTERMINISME DE L’ABONDANCE, DE LA DIVERSITÉ ET DE LA STRUCTURE DES COMMUNAUTÉS MICROBIENNES À DIFFÉRENTES ÉCHELLES SPATIALES	30
4.1 DISTRIBUTION SPATIALE ET DÉTERMINISME DE L’ABONDANCE, DE LA DIVERSITÉ ET DE LA STRUCTURE DES COMMUNAUTÉS MICROBIENNES À L’ÉCHELLE DU TERRITOIRE NATIONAL	30
4.1.1 Abondance des communautés microbiennes des sols	32
4.1.2 Richesse des communautés bactériennes des sols	34
4.1.3 Structure des communautés bactériennes des sols	36
4.1.4 Étude de la TAR	40
4.2 DISTRIBUTION SPATIALE ET DÉTERMINISME DE L’ABONDANCE, DE LA DIVERSITÉ ET DE LA STRUCTURE DES COMMUNAUTÉS MICROBIENNES À L’ÉCHELLE DU PAYSAGE AGRICOLE	45
4.2.1 Abondance des communautés microbiennes des sols	46
4.2.2 Richesse taxonomique des communautés bactériennes des sols	47
4.2.3 Structure des communautés bactériennes des sols	49
4.3 SYNTHÈSE	51
5 DÉVELOPPEMENT ET TRANSFERT D’OUTILS DE DIAGNOSTIC DE LA QUALITÉ MICROBIOLOGIQUE DES SOLS EN APPUI DU DÉVELOPPEMENT AGRICOLE	53
5.1 DÉVELOPPEMENT D’UN OUTIL DE DIAGNOSTIC DE LA QUALITÉ MICROBIOLOGIQUE DES SOLS	53
5.2 TRANSFERT DES OUTILS DE DIAGNOSTIC DE LA QUALITÉ MICROBIOLOGIQUE DES SOLS	57
CHAPITRE 4 – PERSPECTIVES DE RECHERCHE	61
1. PROJET SCIENTIFIQUE DE L’ÉQUIPE BIOCOM	62
2. PROJET DE RECHERCHE AU SEIN DE L’ÉQUIPE BIOCOM	64
2.1 AXE FONDAMENTAL : COMPRENDRE LA DYNAMIQUE SPATIO-TEMPORELLE DE LA DIVERSITÉ MICROBIENNE DES SOLS À L’ÉCHELLE DU PAYSAGE AGRICOLE	65
2.2 AXE APPLIQUÉ : DÉVELOPPER UN OUTIL D’AIDE À LA DÉCISION POUR LA GESTION DES COMMUNAUTÉS MICROBIENNES DES SOLS	67
3. AXE DE TRANSFERT : TRANSFÉRER LES CONNAISSANCES EN ÉCOLOGIE MICROBIENNE DES SOLS ET LES OUTILS ASSOCIÉS POUR LA GESTION DES SOLS	68
3.1 TRANSFERT ACADEMIQUE	68

SOMMAIRE

3.2 TRANSFERT OPERATIONNEL	69
<u>BIBLIOGRAPHIE SÉLECTIVE</u>	<u>79</u>

Chapitre 1 – Parcours Scientifique

1 Historique et positionnement dans l'équipe de recherche

Mes activités de recherche ont toujours été liées à l'étude des sols et de leur fonctionnement. Tout d'abord centrées sur les interactions mycorhiziennes lors de mes premiers stages en laboratoire, je me suis orienté vers le rôle des sols dans le cycle du carbone pendant mon master 2 et surtout pendant mon doctorat intitulé "Les respirations autotrophe et hétérotrophe du sol dans une chênaie tempérée" sous la co-direction de K. Soudani et J.-C. Lata (UMR 8079, Laboratoire Ecologie Systématique Evolution, Université Paris-Sud XI, 2005-2008). Ce doctorat constitue une charnière dans mes activités de recherche car il m'a permis d'ouvrir mes thématiques de recherche vers l'écologie microbienne en initiant une collaboration avec L. Ranjard et P.-A. Maron (UMR Microbiologie du Sol et de l'Environnement, INRA Dijon) pour mieux comprendre les variations d'émissions de CO₂ par les sols. A l'issue de ce doctorat, je me suis orienté un peu plus vers l'écologie microbienne au travers du post-doctorat que j'ai réalisé dans l'équipe BIOCOM (dir. L. Ranjard, DR INRA, UMR 1347 Agroécologie, INRA Dijon) : "Etude de la Biogéographie des communautés de champignons du sol au sein du Réseau de Mesure de la Qualité des Sols" (Direction L. Ranjard, DR INRA, 2008-2010). Ce post-doctorat et mon recrutement en tant que Maître de Conférence à AgroSup Dijon en 2010 ont ancré mon parcours scientifique à Dijon et ont joué un rôle déterminant dans mes thématiques de recherche actuelles que je détaillerai plus loin (Chapitre 2. § 2.2).

Aujourd'hui, mon parcours scientifique se poursuit au sein de l'équipe BIOCOM, dans le pôle BIOME (Biologie et fonctions écosystémiques des sols) de l'UMR 1347 Agroécologie (INRA, AgroSup Dijon, Université de Bourgogne-Franche-Comté). Cette équipe compte 7 personnels permanents : 2 DR, 3 MC, 1 IE, 1 TR ; 1 post-doctorante et 1 PRAG (AgroSup Dijon). L'équipe BIOCOM est structurée autour de 3 axes de recherches (Figure 1) : 1) Etude des processus et des filtres impliqués dans la distribution spatiale des communautés microbiennes du sol à différentes échelles ; 2) Rôle de la diversité microbienne dans le fonctionnement biologique du sol; 3) Diagnostic microbiologique de la qualité des sols agricoles. Mes activités de recherche au sein de l'équipe BIOCOM se déploient à part égale entre les axes 1 et 3. Mon intégration au sein de l'équipe BIOCOM m'a permis d'aller plus loin dans mes activités de scientifiques, en particulier au travers du co-encadrement de la thèse de

F. Constancias (2010-2014) ; mais aussi en termes de responsabilités puisque je suis responsable de l'équipe BIOCUM depuis le 1^{er} Mars 2018.

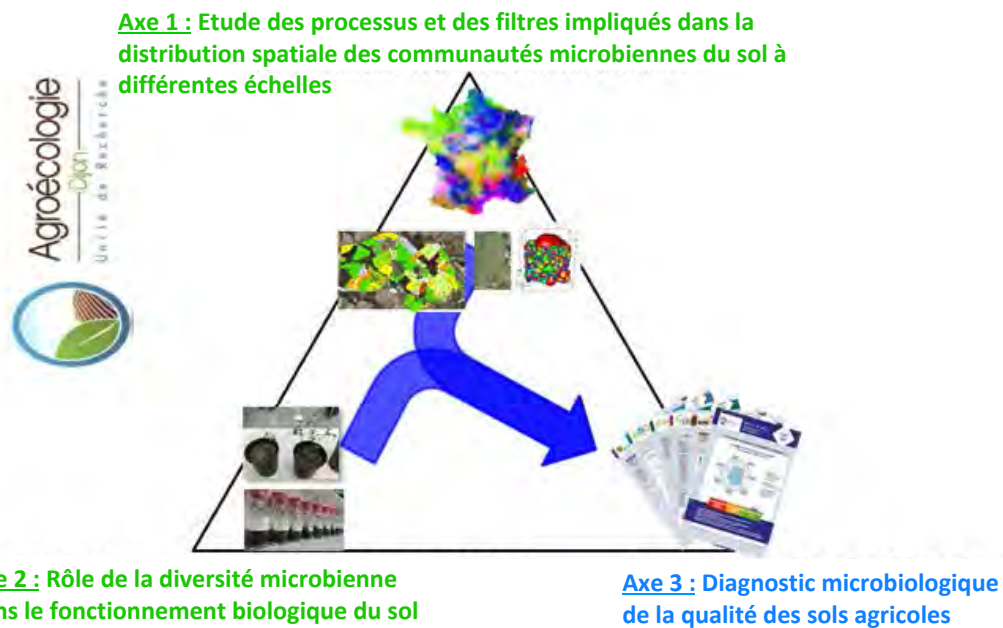


Figure 1. Schéma conceptuel des thématiques de l'équipe BIOCUM et de leurs relations.
Les axes de recherche fondamentale sont identifiés en vert, l'axe de transfert est identifié en bleu.

2 CV

Etat Civil

Nom et Prénom: CHEMIDLIN PREVOST-BOURE Nicolas

Date et lieu de naissance : 30/05/1981, Paris (14e)

Nationalité : Française

Situation familiale : PACSE, 2 enfants

Adresse professionnelle :

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e-mail : nicolas.chemidlin@agrosupdijon.fr

Situation administrative

Depuis le 1^{er} Janvier 2011, Maître de Conférences du Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt (5^e échelon, CNECA N°2)

De septembre 2005 à Janvier 2012, Professeur agrégé classe normale en Sciences de la Vie, Sciences de la Terre et de l'Univers

Formation et cursus professionnel

2011 - ... : **Maître de Conférences** en Biologie des sols – Sciences du sol ; Département Agronomie Agroéquipement Elevage et Environnement ; AgroSup Dijon

2008 – 2011 : **Post-Doctorat** ; UMR Microbiologie du Sol et de l'Environnement, INRA Dijon. *Sujet* : "Etude de la Biogéographie des communautés de champignons du sol au sein du Réseau de Mesure de la Qualité des Sols" ; sous la direction de L. Ranjard

Chapitre 1

2005 – 2008 : Doctorat ; Laboratoire Ecologie – Systématique - Evolution (ESE), Université Paris- Sud XI, Orsay. Mention très honorable et félicitations du jury
Titre: "Les respirations autotrophe et hétérotrophe du sol dans une chênaie tempérée" ; sous la direction de K. Soudani et J.-C. Lata

Composition du Jury :

Ivan JANSSENS, PR, Université d'Anvers, Anvers [rapporteur]

Thierry HEULIN, DR CNRS, CEA Cadarache [rapporteur]

Paul LEADLEY, PR, Université Paris-Sud XI, Orsay [examinateur]

Nathalie FROMIN, CR, CEFE, Montpellier [examinatrice]

Kamel SOUDANI, MCF, Université Paris Sud XI, Orsay [encadrant]

Jean-Christophe LATA, MCF, Université Paris VI, Paris [co-encadrant]

Moniteur à l'Université Paris-Sud XI, Orsay.

2004 – 2005 : 4^e année à l'Ecole Normale Supérieure de Cachan (ENS Cachan)

Master2 Sciences, Technologie, Santé, Mention Ecologie, Biodiversité, Evolution, spécialité Ecologie, Biodiversité, Evolution, Université Paris Sud XI. *Mention bien* Stage de Master 2 : "Les respirations autotrophe et hétérotrophe du sol en forêt tempérée décidue" ; laboratoire ESE sous la direction d'E. Dufrêne, Université Paris Sud-XI.

2003 – 2004 : 3^e année à l'ENS Cachan ; Admis à l'Agrégation SVSTU (81^e)

2002 – 2003 : 2^e année à l'ENS Cachan

Maîtrise de Biologie Cellulaire et Physiologie, mention Génétique Moléculaire et Cellulaire & Physiologie, Université Paris Sud XI. *Mention bien*. Stage de Maîtrise : "Etude de l'effet des bactéries auxiliaires de mycorhization sur la mise en place de mycorhizes in vitro chez des clones de peuplier blanc" ; sous la direction de D. Khasa, Laboratoire de Mycologie, Pavillon Marchand, Université Laval, Québec, Canada

2001 – 2002 : 1^{ère} année à l'ENS Cachan

Licence de Biologie, mention Biologie Cellulaire et Physiologie, Université Paris Sud XI. Mention *Assez Bien*. Stage de Licence : "Contribution à la mise en

Chapitre 1

place d'une technique moléculaire de détection de bactéries intrafongiques chez *Laccaria bicolor* S238N" ; sous la direction de P. Frey-Klett, INRA de Nancy, Centre de Champenoux

1999 – 2001 : Classe préparatoire BCPST au lycée Henri IV (Paris 05). Admis au concours Agro (262e) et aux ENS (68e)

1999 : **Baccalauréat série S** mention Bien, Lycée Richelieu, Rueil-Malmaison

Langues étrangères

Anglais : lu, écrit, parlé (TOEIC : 860) ; *Allemand* : niveau Bac

Formations complémentaires

- Formation "Techniques du génie génétique : PCR en temps réel", 5 jours (29/06/2009 – 03/07/2009), AGROCAMPUS de Rennes.
- Formation "Échantillonnage dans l'espace et dans le temps pour l'inventaire et la surveillance des ressources naturelles, module 1" (23/09/2013 – 27/09/2013), INRA, Orléans.

Animation scientifique et responsabilités

- **Responsabilités au sein d'AgroSup Dijon et de l'UMR Agroécologie**
 - Responsable de l'équipe BIOCUM (1^{er} Mars 2018)
 - Suppléant de Christelle Philippeau au Conseil Scientifique d'AgroSup Dijon (2012-2015)
 - Co-direction de la dominante d'approfondissement « Ressources, Données, Diagnostic, Changements Climatiques » (R2D2C) à AgroSup Dijon (à partir de Septembre 2018)
 - Co-direction de formation diplômante : licence professionnelle "Agriculture, Nouvelles Technologies, Durabilité", Université de Bourgogne Franche-

Chapitre 1

Comté, responsabilité de 4 UE en L3, Master et en formation Ingénieure (depuis 2011)

➤ **Activités d'expertise**

- Expertise de projet dans le cadre des Labex de l'Université Joseph Fourier, Grenoble
- Reviews pour des journaux à comité de lecture de rang A : European Journal of Soil Biology ; Plant and Soil, Geoderma ; Agronomy for Sustainable Development ; Global Change Biology

Projets de recherche

➤ **En tant que coordinateur**

Projets régionaux

- 2011 – 2013 : BIOMAPS : " Biodiversité Microbienne des Sols à l'échelle d'une Mosaïque Agricole : approche écologique et évaluation environnementale des pratiques agricoles à court et moyen terme" – FABER Conseil Régional de Bourgogne
- 2016 – 2018 : Monitoring des communautés microbiennes des sols à l'échelle d'un paysage agricole – Conseil Régional de Bourgogne
- 2016 – 2020 : IFEP : "Impacts de la fertilisation des prairies sur leur biodiversité et sur les transferts de bactéries et de contaminants chimiques du sol au lait" – cofinancement Comité Interprofessionnel de Gestion du Comté / I-Site Bourgogne-Franche-Comté.

Projets nationaux

- 2015 – 2017 : Projet d'édition d'un Atlas microbiologique des sols français – co-coordination avec L. Ranjard – cofinancement ADEME ; Observatoire Français des Sols Vivants ; AgroSup Dijon ; INRA.

➤ **En tant que partenaire**

Projets régionaux

- 2016 – 2018: Eco-dynamiques d'un espace forestier et des activités humaines : la forêt et réserve de Val-Suzon sur le temps long – Conseil

Chapitre 1

Régional de Bourgogne Franche Comté, coordination JP Garcia, Université de Bourgogne Franche Comté

- 2018– 2023 : Réseau de Veille à l'innovation Agricole Bourgogne Franche-Comté – ADEME, UE (FEADER), coordination J. Halska (CA71)

Projets nationaux

- 2006 – 2009 : ECOMIC-RMQS : Microbio-géographie à l'échelle de la France par application d'outils moléculaires au réseau français de mesures de la qualité des sols – ANR, coordination L. Ranjard, INRA
- 2012 – 2016 : MOSAIC : Approche à l'échelle du paysage de la dynamique des Matières Organiques des Sols dans des systèmes Agricoles Intensifs liés à l'élevage, et dans un contexte de Changements globaux – ANR, coordination V. Viaud, INRA
- 2014 – 2018 : CAMMISOLE: Effet du Changement global en Afrique de l'ouest et à Madagascar sur la diversité des Microorganismes du Sol et ses conséquences sur les services Ecosystémiques – FRB, coordination L. Bernard, IRD
- 2011 – 2015: AgrInnov – CASDAR, INRA, AgroSup Dijon, Région Pays de la Loire, Région Bourgogne, ADEME, coordination L. Ranjard, Chef de file : Observatoire Français des Sols vivants.
- 2013 – 2014: META-Taxomic RMQS – France Génomiques, coordination L. Ranjard
- 2011 – 2014 : ECOFINDERS – UE, 6^e PCRD, Coordination P. Lemanceau.
- 2018 – 2022 : Agro-Eco Sol – Projets d'Investissement d'Avenir 3, coordination M. Vallé Aurea AgroSciences, L. Ranjard, INRA et Arvalis institut du végétal.

Collaborations scientifiques

- UMR Chronoenvironnement (F Gillet, D Gilbert, PM Badot)
- UMR CNRS 5558, Laboratoire de Biométrie (J Thioulouse)
- Unité Infosol (D Arrouays, C Jolivet, N Saby, INRA Orléans)
- UMR SAS (V Viaud, INRA rennes)

Chapitre 1

- UMI 209 UMMISCO (N Marilleau, IRD)
- UMR 6282 Biogeoscience (J Lévêque, O Mathieu, Université de Bourgogne Franche-Comté)
- UMR 1347 Agroécologie AgroSup Dijon, INRA, Université de Bourgogne Franche-Comté (S Petit, F Dessaint, JP Guillemin)
- ADEME (A Bispo)
- Comité Interprofessionnel de Gestion du Comté (Y Bouton)
- Chambres d'Agriculture (P Mulliez et V Riou (49), J Halska (71))
- Observatoire Français des Sols Vivants (E. d'Oiron)

Activités de formation en lien avec la recherche et responsabilités

➤ Formation académique de la licence au doctorat

- **Co-direction de la dominante d'approfondissement « Ressources, Données, Diagnostic, Changements Climatiques » (R2D2C) à AgroSup Dijon** (à partir de Septembre 2018). Elle vise à former des ingénieurs opérationnels capables de proposer des stratégies de gestion des ressources naturelles (eau, sol, biodiversité) en contexte de changement climatique. Elle s'appuie sur une démarche en trois temps : 1) évaluation de l'état des ressources, 2) diagnostic, 3) Proposition d'actions ; en mettant en œuvre des outils de diagnostic éprouvés, en particulier les outils de diagnostic microbiologique des sols issus de mes activités de recherche finalisée.
- **Co-direction de formation diplômante : licence professionnelle "Agriculture, Nouvelles Technologies, Durabilité", Université de Bourgogne Franche-Comté, responsabilité de 4 UE en L3, Master et en formation Ingénieure** (depuis 2011)
- **Encadrement de stages recherche :**
 - Licence et Master : S. Aussems (L3Pro, 2010), Cécile Gruet (2017, L3) ; Rémy Doyen (2009, M2, co-direction L. Ranjard) ; Dewi Gleau

Chapitre 1

(2013, M2, co-direction : JP Guillemain), Emmanuelle Franc (M2 Pro, 2016, co-direction).

- Thèse de doctorat : F. Constancias : "Distribution spatiale de la diversité bactérienne tellurique à différentes échelles spatiales : de l'agrégat au paysage" (2011 à 2015, co-direction L. Ranjard).
- Post-doctorat : Battle Karimi : "Atlas français des bactéries du sol" (2015-2017, co-encadrement avec L. Ranjard) ; Battle Karimi (2017-2018)

➤ **Formation professionnelle**

- **Formations à destination des agriculteurs et des viticulteurs :**

"Les indicateurs biologiques et agronomiques de la qualité des sols" : 13 formations de deux jours depuis 2013.

- **Formations à destination des conseillers agricoles (RESOLIA) :**

"Les indicateurs biologiques et agronomiques de la qualité des sols" : 4 formations de 3 jours depuis 2015 dont 3 en tant qu'intervenant et 1 en tant que responsable de formation.

- **Formations à destination des enseignants des lycées agricoles (Plan National de Formation du Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt):**

1 formation d'une journée et demie en 2016.

Liste des travaux

24 articles dans des revues à comité de lecture, 5 chapitres d'ouvrage et 1 ouvrage en cours de publication; H index =10

➤ **Articles soumis dans des revues à comité de lecture**

Karimi B., Dequiedt S., Terrat S., Jolivet C., Arrouays D., Wincker P., Cruaud C., Bispo A., Chemidlin Prévost-Bouré N., Ranjard L. (2018). Soil Bacterial Networks Driven by Land Use on Broad-scale, *Soil Biology and Biochemistry*, *soumis*

Karimi B., Terrat S., Dequiedt S., Saby N.P.A., Horrigue W., Lelièvre M., Nowak V., Jolivet C., Arrouays D., Wincker P., Cruaud C., Bispo A., Maron P.-A., Chemidlin

Prévost-Bouré N., Ranjard L. (2018). Biogeography of Soil Bacteria and Archaea across France, *Science Advances*, *in press*.

Le Guillou C., Chemidlin Prévost-Bouré N., Akkal-Corfini N., Dequiedt S., Nowak V., Terrat S., Menasseri-Aubry S., Viaud V., Maron P.-A., Ranjard L. (2018). Tillage intensity and pasture in rotation effectively shape soil microbial communities at a landscape scale, *MicrobiologyOpen*, *soumis*.

➤ **Articles publiés dans des revues à comité de lecture**

[1] Sadet-Bourgeteau S., Houot S., Dequiedt S., Nowak V., Tardy V., Terrat S., Montenach D., Mercier V., Karimi B., Chemidlin Prévost-Bouré N., Maron P.-A. (2018). Lasting effect of repeated application of organic waste products on microbial communities in arable soils, *Applied Soil Ecology*, *in press*.

[2] Bernard L., Razanamalala K., Razafimbelo T., Maron P.-A., Ranjard L., Chemidlin Prévost-Bouré N., Lelievre M., Dequiedt S., Ramaroson V., Becquer T., Trap J., Blanchart E., Marsden C. (2017). "Soil microbial diversity drives the priming effect along climate gradients – A case study in Madagascar", *ISME Journal*, doi:10.1038/ismej.2017.178

[3] Terrat S., Horrigue W., Dequiedt S., Saby N.P.A., Lelièvre M., Nowak V., Tripied J., Regnier T., Jolivet C., Arrouays D., Wincker P., Cruaud C., Karimi B., Bispo A., Maron P.-A., Chemidlin Prévost-Bouré N., Ranjard L. (2017). Mapping and Predictive Variations of Soil Bacterial Richness across France, *Plos One*, 12, 10.

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➤ **Communications dans des congrès avec publication des actes**

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Chemidlin-Prévost-Bouré N. (2008) Les respirations autotrophe et hétérotrophe du sol dans une chênaie tempérée. *Thèse de Doct. en Sci., Univ Paris-Sud, Orsay*, 220 p

➤ Autres productions : Déclarations d'invention

- [1] **DIRV-16-0042** : Diagnostic de la qualité microbiologique d'un sol par la mesure de la biomasse moléculaire microbienne, Ranjard L., Dequiedt S., Maron P.A., Horrigue W., Chemidlin Prévost-Bouré N.
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Chapitre 2 – Synthèse des activités de recherche

1 Introduction

Le fonctionnement [biologique] des sols occupe une part primordiale pour les activités humaines. Au travers de leurs propriétés, les sols déterminent notre capacité à produire des biens en quantité et en qualité. Dans ce contexte, la composante microbienne des sols occupe une place prépondérante. Le sol héberge une extrême diversité de microorganismes. Ils sont les plus abondants : un gramme de sol contient environ 10^9 bactéries et 10^5 champignons ($\approx 50\%$ de la biomasse du sol, $\approx 5t$ de Carbone par hectare ; Maron *et al.*, 2011) ; et les plus diversifiés : environ 10^6 espèces bactériennes et 10^3 à 10^4 espèces de champignons par gramme de sol (Torsvik, 2002; Bates *et al.*, 2013). Cette extrême diversité taxonomique des microorganismes du sol supporte une extraordinaire diversité fonctionnelle qui leur confère un rôle très important dans le fonctionnement biologique des sols. D'un point de vue qualitatif, ils sont impliqués dans tous les cycles biogéochimiques (Carbone, Azote, Phosphore ; Chemidlin Prevost-Boure *et al.*, 2010b) ; le maintien de la structure du sol ; la dégradation des composés exogènes (e.g. pesticides) ou réduisent la durée de vie de certains pathogènes dans le sol (Vivant *et al.*, 2013). D'un point de vue quantitatif, l'intensité de certaines fonctions dépend du niveau de diversité de la communauté (e.g. minéralisation de la MO et dénitrification ; Philippot *et al.*, 2013; Tardy *et al.*, 2014). En plus de cette importance stratégique pour le fonctionnement du sol, la diversité des communautés microbiennes (richesse spécifique, composition taxonomique) détermine tout ou partie du niveau de redondance de ces fonctions et donc leur stabilité (résistance/résilience) en réponse à des perturbations (pratiques agricoles, changements climatiques). Les communautés microbiennes constituent donc une assurance écologique pour l'Homme (Loreau, 2010).

Néanmoins, l'utilisation intensive des sols les soumet à des pressions croissantes conduisant à la dégradation de cette ressource naturelle dont les effets se ressentent aujourd'hui, notamment au niveau agricole. En effet, historiquement adossée à des leviers externes à l'agroécosystème pour garantir des rendements élevés, l'agriculture connaît actuellement une transition forte vers l'agroécologie (ensemble d'agricultures alternatives à l'agriculture dont le système de production n'est adossé qu'à des leviers externes à l'agroécosystème ; *in* [l'Agroévolution Française](#), Vincent Tardieu, 2012) pour favoriser la durabilité des exploitations agricoles. Ainsi, le sol

Chapitre 2

n'est plus un support de productions mais un écosystème "piloté de manière à fournir durablement diverses catégories de biens et de services précisément qualifiés" (Millenium Ecosystem Assessment, 2010). Dans ce contexte, l'enjeu est donc d'être en mesure d'utiliser au mieux les composantes biologiques des sols, et particulièrement les communautés microbiennes au regard de leur importance quantitative et qualitative. Pour cela, il est nécessaire de mieux comprendre les déterminants de la diversité des communautés microbiennes des sols ; et de diffuser les connaissances et les outils de gestion auprès des acteurs de terrain, en particulier du monde agricole.

L'écologie microbienne évolue fortement depuis 20 ans avec le développement et l'amélioration continue des techniques d'écologie moléculaires basées sur l'étude du métagénôme du sol. Elles ont progressivement permis de caractériser à haut débit et de plus en plus finement la diversité microbienne des sols tant en termes de richesse taxonomique (diversité α) ou de structure des communautés microbiennes (abondance relative des différents taxons présents) ; offrant ainsi la possibilité de cartographier la diversité des microorganismes du sol. Elles sont aussi à l'origine d'évolutions fondamentales indispensables à la compréhension de l'écologie des microorganismes du sol. Leur mise en œuvre a permis de démontrer que les approches biogéographiques (*i.e.* l'étude de la distribution des organismes dans l'espace et le temps) historiquement inféodées aux plantes et aux macroorganismes étaient applicables aux microorganismes du sol (Horner-Devine *et al.*, 2004). Ceci permet aujourd'hui d'évaluer si les microorganismes du sol ont une écologie particulière comme le suggère le postulat de Baas Becking (1934) : "tout est partout, mais l'environnement sélectionne" ; ou s'ils sont soumis aux mêmes lois écologiques que les macroorganismes et les plantes. D'une part, l'association des techniques d'écologie moléculaire aux approches biogéographiques permet d'évaluer si les communautés microbiennes des sols suivent une des plus robustes et plus anciennes relations en écologie : la relation Aire-Taxons (TAR, augmentation du nombre de taxons observés avec l'aire d'échantillonnage ; Arrhenius 1921; Gleason 1922) et d'évaluer le postulat de Baas-Becking. D'autre part, l'étude des variations spatiales de structure des communautés microbiennes des sols (diversité β) permet dans un premier temps d'évaluer si les communautés microbiennes des sols sont soumises aux même processus écologiques que les macroorganismes : la sélection

Chapitre 2

environnementale, la dispersion (*i.e.* le déplacement et l'établissement avec succès d'un organisme dans un nouveau lieu), la dérive écologique (*i.e.* des variations d'abondance relative des différents taxons de la communauté suite à différents évènements démographiques) et la mutation (*i.e.* l'apparition de nouvelles espèces suite à des mutations génétiques). Dans un second temps, elle permet d'identifier et de quantifier clairement ces processus pour les hiérarchiser, mais aussi d'identifier et de hiérarchiser les filtres environnementaux supportant ces processus : caractéristiques physico-chimiques du sol, occupation du sol, pratiques agricoles, localisation géographique. Ceci permet alors d'identifier de potentiels leviers de gestion des communautés microbiennes des sols. Par ailleurs, comme les macroorganismes, les microorganismes constituant les communautés microbiennes interagissent entre eux et il est aujourd'hui possible de rendre compte de ces interactions dans la compréhension de l'écologie des microorganismes du sol.

Dans la suite, je présenterai ma thématique, mes objectifs et ma stratégie de recherche. Puis, je présenterai mes activités de recherche fondamentale au travers de trois parties couvrant ma thèse, mes travaux de post-doctorat à l'échelle du territoire national et mes travaux à l'échelle d'un paysage agricole. Une dernière partie me permettra de décrire mes travaux de recherche finalisée et leur mise en œuvre dans le cadre d'activités de recherche participative en lien avec le développement agricole.

2 Thématique, objectifs et stratégie de recherche

2.1 Thématique de recherche et objectifs

Ma thématique de recherche est la biogéographie microbienne des sols à l'échelle du territoire national et des paysages agricoles ; la biogéographie étant l'étude de la distribution des organismes dans l'espace et le temps. Je la décline au sein de deux des axes de recherche de l'équipe BIOCOM, l'étude du déterminisme spatial et temporel des communautés microbiennes du sol et le diagnostic microbiologique des sols agricoles. Dans l'axe 1, mes objectifs de recherche sont de: 1) décrire la distribution spatiale des communautés bactériennes et fongiques, 2) évaluer les processus écologiques et leur intensité au travers de la relation "aire - espèces" (augmentation du nombre d'espèces inventoriées avec l'augmentation de l'aire d'échantillonnage), 3) hiérarchiser les filtres environnementaux déterminant ces variations, 4) évaluer l'impact du mode d'usage des sols sur ces communautés. Au sein de l'axe 3, mon objectif est d'accélérer le transfert des outils de diagnostic et des connaissances associées pour l'évaluation des pratiques auprès des acteurs du monde agricole (agriculteurs, conseillers de chambre d'agriculture, interprofessions...) en appui du développement agricole pour la transition agroécologique.

2.2 Stratégie de recherche

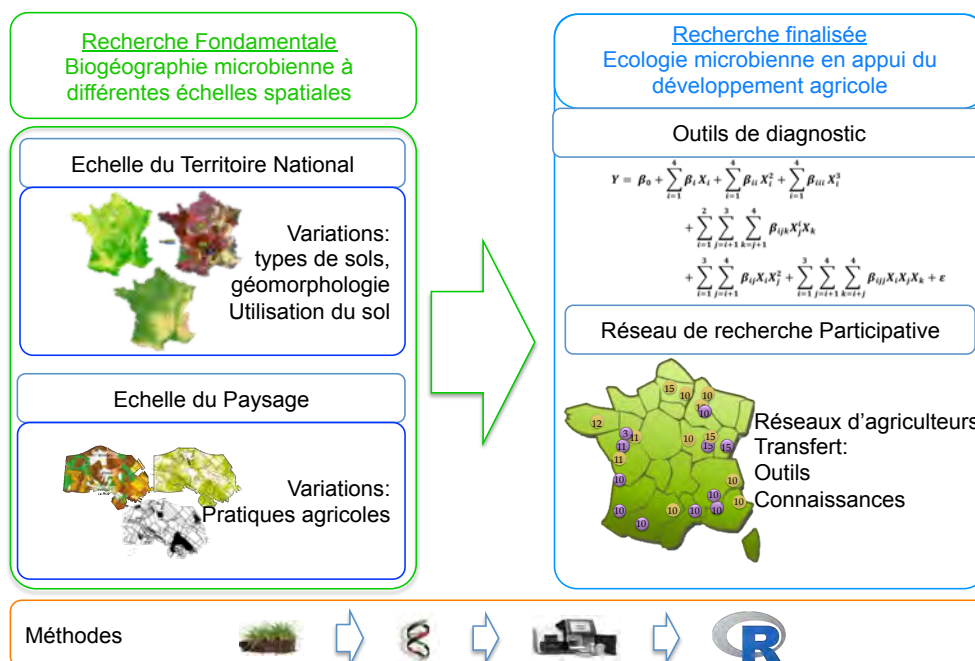


Figure 2. Stratégie de recherche.

Le schéma décrit le continuum entre recherche fondamentale et recherche finalisée en mettant en évidence les échelles investiguées et les sorties finalisées en termes d'outils de diagnostic et de recherche participative. Le cadre méthodes identifie la séquence technique mise en œuvre aux différentes échelles spatiales : extraction d'ADN du sol, séquençage des communautés microbiennes et analyses statistiques et dans les approches de recherche finalisée.

Pour atteindre ces objectifs, ma stratégie de recherche met en jeu des approches à différentes échelles spatiales (Territoire national et paysage agricole) et de métagénomique environnementale pour considérer la réponse des communautés microbiennes des sols à différents types de variations environnementales : caractéristiques des sols, géomorphologie, pratiques agricoles (Figure 2). Ainsi, il est possible de mieux comprendre la biogéographie des communautés microbiennes des sols en les cartographiant et en identifiant les processus et les filtres environnementaux déterminant leurs variations. La comparaison des résultats entre échelles permet d'évaluer la généralité des résultats à différentes échelles spatiales.

Les connaissances acquises permettent ensuite de développer des outils de diagnostic d'impact des pratiques agricoles opérationnels et transférables aux acteurs du monde agricole (ADEME, Observatoire National de la Biodiversité) dans le cadre d'une recherche finalisée. Je déploie ces outils dans des projets de recherche participative incluant plusieurs acteurs du monde agricole (agriculteurs, conseillers de chambre d'agriculture...) permettant ainsi d'accompagner la transition agroécologique.

3 Étude des émissions de Carbone par les sols en forêt tempérée décidue

Cette partie s'appuie en particulier sur ma thèse de doctorat et les articles référencés sous les numéros [18], [19] & [23].

J'ai réalisé mon doctorat sur les émissions de carbone (C) par le sol en milieu forestier, généralement nommée respiration du sol. Mes travaux s'inscrivaient dans un contexte de changements climatiques où la compréhension du fonctionnement du sol est cruciale puisque la respiration du sol (R_S) représente entre 40% et 70% des émissions de CO_2 par les écosystèmes continentaux, déterminant ainsi leur l'état de source ou de puits de C. Plus précisément, mes travaux portaient sur l'étude des deux composantes autotrophe (R_A) et hétérotrophe (R_H) de R_S afin de mieux comprendre les processus impliqués dans le fonctionnement du sol et leurs réponses faces aux variations des facteurs environnementaux.

J'avais pour objectifs de décomposer R_S en ses composantes R_A et R_H d'une part ; et de déterminer l'effet de variations de facteurs biotiques et abiotiques sur le fonctionnement du sol d'autre part. J'ai atteint ces objectifs au travers de deux expérimentations de terrain réalisées dans une forêt tempérée décidue (forêt de Barbeau, site FR-Font, Réseau CarboEurope-IP).

La décomposition de R_S en R_A et R_H a mis en jeu la méthode des « Trench plot » couplée à des mesures de R_S et l'utilisation des isotopes stables du C en abondance naturelle. Ce couplage entre différentes techniques a permis de tester l'impact de l'exclusion racinaire sur la composition isotopique du CO_2 sortant du sol ($\delta^{13}C_{R_S}$). Les résultats ont permis de montrer que R_H contribuait à hauteur de 40 à 60% de R_S (Figure 3). L'étude des déterminants de R_S montre qu'elle augmente avec la température du sol ; cette augmentation étant modulée par l'humidité du sol suivant une loi lognormale présentant un optimum à la capacité au champ. Au contraire, l'exclusion racinaire n'a pas affecté $\delta^{13}C_{R_S}$, ce qui laisse supposer que la différence de composition isotopique entre R_A et R_H est trop faible pour décomposer précisément R_S dans des écosystèmes forestiers sans alternance entre types photosynthétiques C3 et C4.

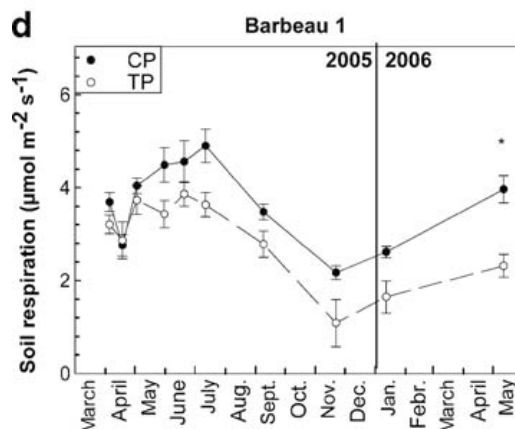


Figure 3. Dynamique temporelle de la respiration du sol dans le trench plot (TP) et le plot contrôle (CP).

Les barres d'erreur correspondent à des erreurs standard et les points correspondent aux respirations moyennes par plot (n=30). Article associé : [23].

Les effets associés aux variations de facteurs biotiques sur le fonctionnement du sol ont été étudiés *via* une modification quantitative des allocations de litière foliaire au sol (apport normal, apport doublé, pas d'apport), et par un suivi mensuel de R_S , de $\delta^{13}\text{C}_{RS}$ et de la structure des communautés bactériennes (BCS) du sol et de la litière.

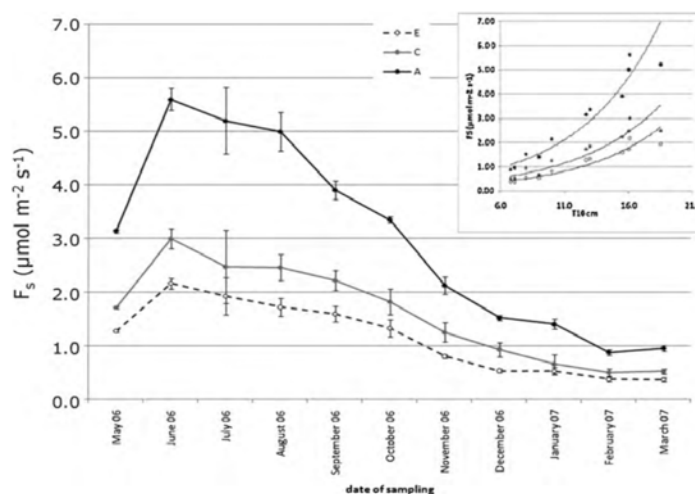


Figure 4. Dynamique temporelle de la respiration du sol (F_S) dans les différentes modalités d'apport de litière.

Les lignes noires, grises et en pointillés correspondent aux modalités d'apport de litière : apport doublé, apport normal et pas d'apport ; respectivement. Les barres d'erreur sont des erreurs standards. L'encart en haut à droite montre la relation entre la température du sol et l'intensité de la respiration du sol. Article associé [19]

Les résultats (Figure 4) montrent que R_S augmente de façon non proportionnelle à la quantité de litière allouée. Cette augmentation a été reliée à un « priming effect » (PE). L'intensité des flux de CO_2 issus du sol de surface et de la litière n'ayant pas

été affectée par le traitement, le PE a été localisé dans du sol profond et comme utilisant du C récalcitrant. Ceci pourrait être confirmé par les mesures de $\delta^{13}\text{C}_{\text{Rs}}$ mais ces dernières n'ont pas été affectées par le traitement, la signature isotopique de la matière organique du sol étant relativement proche dans les horizons profonds de celle de l'horizon de surface. Néanmoins, les BCS du sol et de la litière ont été significativement modifiées par le traitement, mettant en avant de potentielles variations de fonctionnement du sol, hypothèse qui s'est vue appuyées par la mise en évidence d'un lien statistique entre les variations de BCS et l'intensité et la composition isotopique du flux de CO_2 dans la litière.

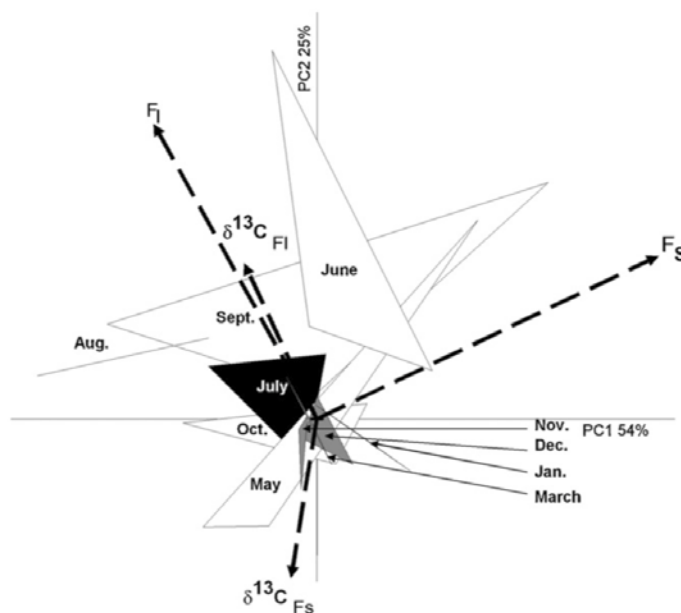


Figure 5. Analyse de coinertie entre l'évolution de la structure des communautés bactériennes de la litière et l'intensité et la signature isotopique de la respiration du sol (F_S , $\delta^{13}\text{C}_{F_S}$) ou de la litière (F_L , $\delta^{13}\text{C}_{F_L}$).

Les axes de la coinertie prennent en compte 79% des variations de structure des communautés bactériennes et de l'intensité/de la signature isotopique des flux de CO_2 . Les polygones correspondent à des convexes de Hull dont la taille est proportionnelle à la variabilité des BCS. Les polygones en gris correspondent aux mois où la température du sol est descendue en dessous de 10°C et le polygone noir indique une sécheresse estivale. Les flèches sont proportionnelles à la corrélation entre les axes de la coinertie et l'intensité/de la signature isotopique des flux de CO_2 . Lorsque les flèches se superposent avec les convexes de Hull, il y a un lien statistique entre les deux. La significativité de ce lien a été testée par un test de Monté-Carlo (1000 permutations). Article associé [18].

L'ensemble de ces résultats a permis de mettre en évidence l'importance d'études intégrées de la R_S . En particulier, pour mieux comprendre le fonctionnement du sol et ses variations dans un contexte de changements climatiques, il apparaît nécessaire de prendre en compte à la fois les variations du flux de CO_2 sortant du sol en lien avec les paramètres abiotiques du milieu (température, humidité), mais aussi la variabilité du système sol en termes de structure des communautés microbiennes.

4 Distribution spatiale et déterminisme de l'abondance, de la diversité et de la structure des communautés microbiennes à différentes échelles spatiales

Cette partie s'appuie en particulier sur les articles référencés sous les numéros [3-5], [7-10], [12], [14-15] & [22].

Cette partie vise à synthétiser mes travaux en biogéographie microbienne des sols à deux échelles d'étude différentes : le territoire national et le paysage agricole. Aux deux échelles, les objectifs étaient de : 1) décrire la distribution spatiale des communautés bactériennes et fongiques ; 2) évaluer l'importance relative de différents processus écologiques (déterministe [Sélection environnementale] vs neutres [dispersion]) structurant les communautés microbiennes du sol ; 3) identifier et hiérarchiser les filtres environnementaux supportant ces processus : physico-chimie du sol, climat, utilisation du sol ou pratiques agricoles (travail du sol, amendements organiques...). Dans la suite, je présenterai les résultats à l'échelle du territoire national puis à celle du paysage agricole. Je ferai ensuite une synthèse entre échelles.

4.1 Distribution spatiale et déterminisme de l'abondance, de la diversité et de la structure des communautés microbiennes à l'échelle du territoire national

Les études à l'échelle du territoire national s'appuient sur le Réseau de Mesure de la Qualité des Sols (RMQS ; Arrouays et al., 2002 ; [22]). Mis en place en 2001 par le GISSOL et coordonné par l'INRA (Unité InfoSol, Orléans), le RMQS est un réseau de surveillance à long terme (cyclicité de 10 à 15 ans) de la qualité des sols en France. Il compte 2200 sites répartis suivant une grille systématique de 16km de côté et est représentatif de la diversité des types de sols et des modes d'usage des sols à l'échelle de la France (Figure 6).

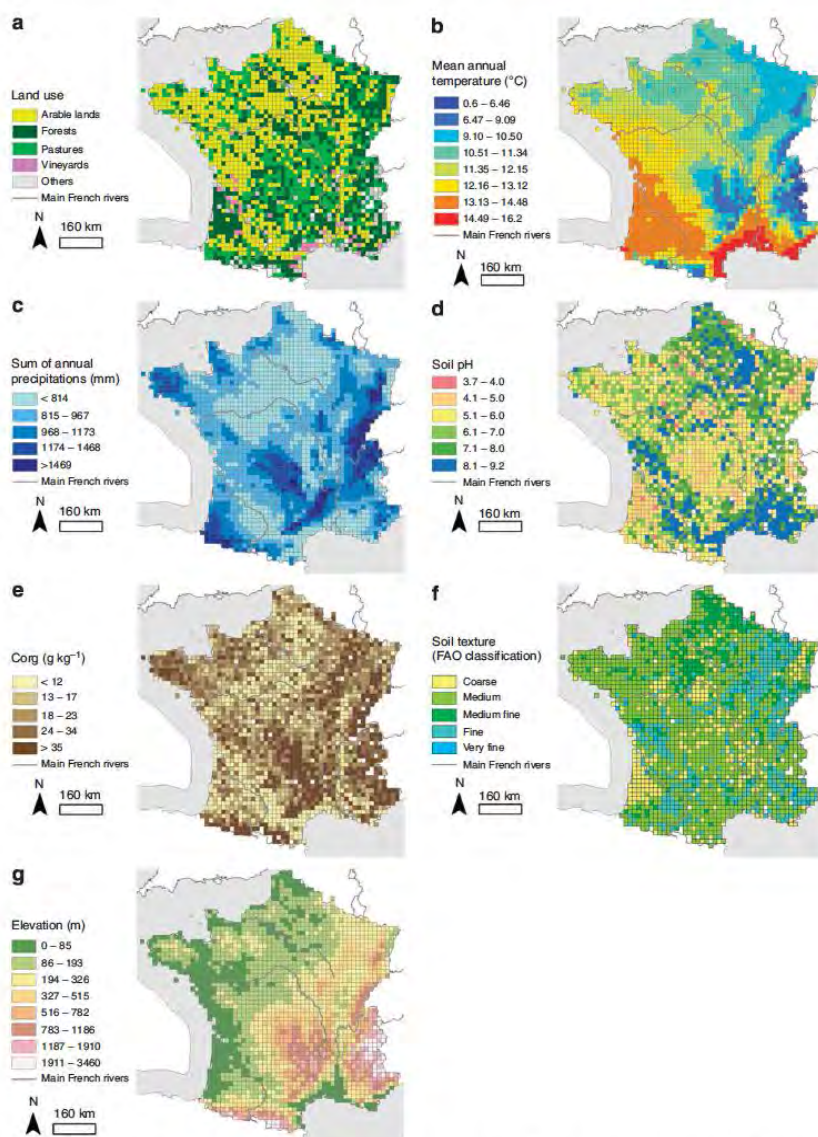


Figure 6. Variabilité environnementale à l'échelle de la France.

Chaque pixel correspond à un site RMQS. Les différentes variables représentées sont : (a) l'utilisation du sol sur la base du Corinne Land Cover niveau 1 ; (b) La température annuelle moyenne ; (c) la somme des précipitations annuelles ; (d) le pH du sol ; (e) la teneur en carbone organique du sol ; (f) la texture du sol au regard de la classification de la Food and Agricultural Organisation des Nations Unies (FAO) ; (g) l'altitude de chaque site d'après les modèles numériques de terrain de l'IGN.

La première campagne du RMQS a duré près de 12 ans et l'étude de la biogéographie des communautés microbiennes s'est déroulée sur les 10 dernières années environ dans le cadre du programme ECOMIC-RMQS (ANR, Coordination L. Ranjard). Sachant que les méthodes de métagénomique connaissent des améliorations techniques tous les 3 à 5 ans, l'étude de la biogéographie des communautés microbiennes a mis en œuvre plusieurs méthodes de métagénomique différentes. Dans chaque partie, je décrirai donc brièvement la méthode mise en œuvre.

4.1.1 Abondance des communautés microbiennes des sols

Articles [5] & [22]

Plusieurs méthodes existent pour mesurer la biomasse microbienne d'un sol. En particulier, la quantité d'ADN directement extraite d'un échantillon de sols est reconnue comme une mesure robuste et reproductible de la biomasse microbienne d'un sol (Dequiedt *et al.*, 2011) et a été nommée biomasse moléculaire microbienne pour la différencier des mesures de biomasse microbienne s'appuyant sur une quantification du carbone ou de l'azote microbien.

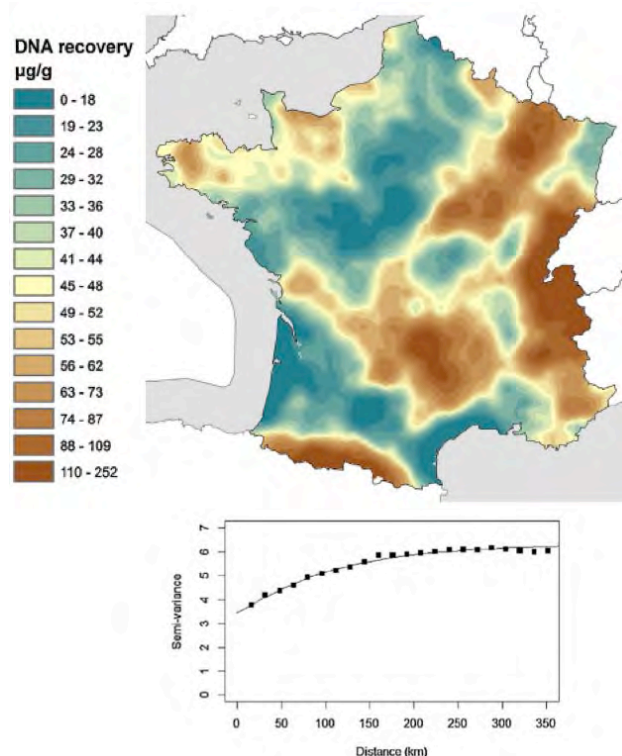


Figure 7. Cartographie de la biomasse moléculaire microbienne des sols à l'échelle du territoire national. En dessous de la carte, le variogramme utilisé pour réaliser l'interpolation est proposé. L'article associé est référencé sous le numéro [5].

L'abondance des communautés microbiennes des sols à l'échelle du territoire national varie entre 0.1 et 630 $\mu\text{g}_{\text{ADN}}\cdot\text{g}_{\text{sol}}^{-1}$ (Figure 7). Cette gamme de variations est du même ordre de grandeur que celles observées dans d'autres études à des échelles similaires ou plus larges (Zhou *et al.*, 1996, Kuske *et al.*, 1998, Frostegard *et al.*, 1999, Ranjard *et al.*, 2003; Serna-Chavez *et al.*, 2013). Ces variations de biomasse moléculaire microbienne du sol sont distribuées de manière hétérogène et structurée, la relation entre la semi-variance et la distance représentée par le variogramme étant significative. La taille des unités spatiales à l'échelle du territoire

Chapitre 2

français est d'environ 160km de rayon, ce qui est très important au regard de la taille des microorganismes du sol. Cette structuration spatiale à plusieurs échelles est en accord avec la bibliographie puisqu'elle est observée à l'échelle globale (Serna-Chavez *et al.*, 2013; Fierer, 2017). Néanmoins, cette structuration spatiale présente aussi une forte variabilité locale au regard du fort effet pépite du variogramme de la biomasse moléculaire microbienne (ca. 50%). L'échelle spatiale de ces variations est sans doute inférieure à la résolution de la carte (ici, 2,5km).

Une approche de partition de la variance de la biomasse moléculaire microbienne mettant en œuvre une analyse canonique des redondances a permis d'identifier et de hiérarchiser les filtres environnementaux impliqués. Les facteurs les plus importants pour expliquer les variations de biomasse moléculaire microbienne sont la teneur en Carbone organique du sol (C_{org} (g/kg) : 15.4%) et l'utilisation du sol (2.9%), indépendamment des interactions potentielles entre ces deux variables. Ceci est en accord avec les observations de la littérature (Serna-Chavez *et al.*, 2013, Ranjard *et al.*, 2003). L'effet positif du C_{org} est lié à son statut de ressource trophique pour tous les microorganismes du sol. L'effet de l'utilisation du sol à l'échelle du territoire national peut être associé à l'intensité d'utilisation du sol : dans les systèmes forestiers et prairiaux, le sol est soumis à une pression anthropique inférieure à celle des systèmes en grande culture et des systèmes horticoles et viticoles dans lesquels il est travaillé de manière plus ou moins forte au moins une fois par an et connaît des apports plus ou moins répétés de nutriments et de produits phytosanitaires. Le second groupe de filtres environnementaux déterminant le niveau de biomasse moléculaire microbienne correspond à un ensemble de conditions réactionnelles : le pH (1.0%), la teneur en argiles (1.9%) et en carbonates (0.9%), le ratio C/N (0.7%), la teneur en potassium (0.5%) et la température annuelle moyenne (0.8%). Le pH et la teneur en argiles ont un effet positif. Le pH détermine le niveau d'activité enzymatique des microorganismes (Fierer, 2017) et donc les capacités de dégradation de la matière organique par les microorganismes du sol. L'effet positif des argiles s'explique par leur rôle de protection de la matière organique et des microorganismes, permettant ainsi de protéger les microorganismes de leurs prédateurs tout en limitant l'accessibilité de la matière organique aux autres organismes [12]. Le ratio C/N et la teneur en carbonates ($CaCO_3$) ont un effet négatif sur la biomasse moléculaire microbienne, notamment car une augmentation de ratio

C/N traduit une diminution de la dégradabilité de la matière organique, et une augmentation de la teneur en carbonates dans un sol augmente le niveau de protection de la matière organique.

4.1.2 Richesse des communautés bactériennes des sols

Article [3]

Les communautés microbiennes des sols sont composées d'une grande diversité de microorganismes appartenant à différents règnes : Bactéries, Archées et Champignons, notamment. Elles peuvent être caractérisées par différentes métriques telles que la richesse taxonomique (diversité α) ou la structure de la communauté. Ici, je me focaliserai sur la richesse taxonomique des communautés bactériennes du sol. Ceci a été rendu par le déploiement de méthodes de séquençage haut débit sur l'ensemble du RMQS (France Génomiques, programme Méta-TAXOMIC RMQS, Coordination L. Ranjard). Ces méthodes se sont démocratisées dans les années 2010 (Check Hayden, 2014) et sont reconnues efficaces pour caractériser les communautés microbiennes des sols (Cuny, Maron & Ranjard, 2017). Les communautés bactériennes sont caractérisées à partir de l'ADN directement extrait du sol à l'aide de marqueurs taxonomiques spécifiques des bactéries (ADNr 16S, ici : F479 (5'-CAGCMGCYGCNGTAANAC-3') et R888 (5'-CCGYCAATTCMTTTRAGT-3')). L'analyse bioinformatique des séquences ([3] & [9]) permet de clusteriser les 10 000 séquences obtenues par échantillon en Unités Taxonomiques Opérationnelles (OTU) à 95% de similarité génétique, ce qui correspond à l'échelle du genre. Le nombre d'OTUs identifiées par échantillon constitue la mesure de richesse bactérienne.

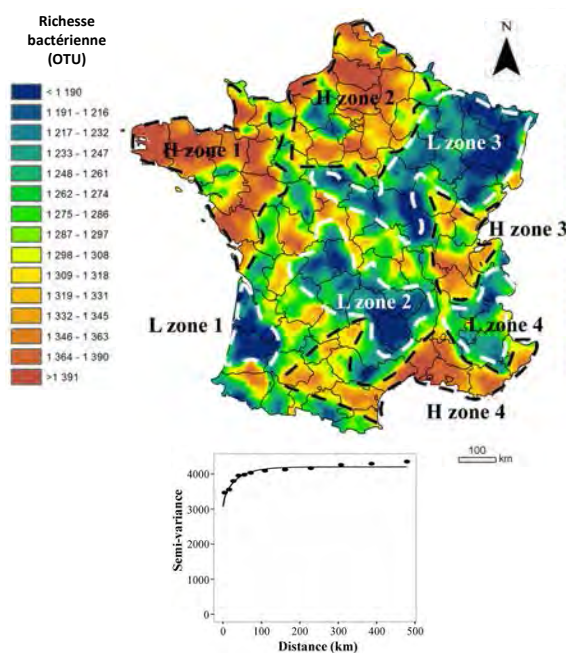


Figure 8. Cartographie de la richesse bactérienne des sols à l'échelle du territoire national
 Le variogramme sur lequel s'appuie l'interpolation est présenté en dessous de la carte. Les zones détournées mettent en évidence des zones présentant des niveaux de diversité forts (H-zone) ou faibles (L-zone). L'article associé est référencé sous le numéro [3].

La Figure 8 présente la distribution spatiale de la richesse bactérienne à l'échelle du territoire national. La gamme de variation est assez large : 555 à 2007 OTU par échantillon de sol. Elle est en accord avec la bibliographie puisque des études à des échelles globales comparables en termes de profondeur de séquençage ont montré une richesse bactérienne variant de 500 à 4400 OTU par échantillon de sol (Delgado - Baquerizo *et al.*, 2017; Thompson *et al.*, 2017; Delgado-Baquerizo *et al.*, 2018). L'étude des variations spatiales de la richesse bactérienne des sols démontre sa distribution hétérogène et structurée en unités spatiales de 110km de rayon environ. Néanmoins, comme pour la biomasse moléculaire microbienne, le fort effet pépite (73%) montre qu'elle varie aussi fortement à des échelles plus fines.

En mettant en œuvre la même approche de partition de la variance, les filtres environnementaux déterminant la richesse taxonomique des communautés bactériennes des sols ont pu être identifiés et hiérarchisés. Il ressort de cette analyse que la richesse taxonomique bactérienne est particulièrement sous la dépendance du pH (11.0%, effet positif), de la teneur en argiles (5.8%, effet négatif) et de l'utilisation du sol (1.4%). L'effet positif du pH est lié à son rôle dans l'accessibilité à l'ensemble des ressources du sol (carbone, azote, conditions réactionnelles ; Fierer, 2017). Ainsi, il détermine en partie la diversité des niches écologiques dans le sol.

De même, la diversité des niches écologiques est affectée par la teneur en argile : son augmentation réduit l'hétérogénéité de l'environnement à micro-échelle, limitant alors la diversité des niches écologiques (Chau *et al.*, 2011). Le troisième filtre est l'utilisation du sol. Fait surprenant, les systèmes les plus perturbés (grandes cultures, vignes et vergers, systèmes en labour) présentent les niveaux de richesse bactérienne les plus élevés par rapport à des systèmes plus stables (forêts, prairies). Ces différences s'expliquent bien par la théorie des perturbations intermédiaires dans laquelle une perturbation modérée diminue la compétitivité des organismes les plus adaptés à un environnement donné, permettant ainsi à d'autres espèces, moins adaptées au milieu initial, de s'implanter et de se développer. Pour les bactéries du sol, les systèmes viticoles, horticoles et de grandes cultures sont modérément perturbés au regard de leur petite taille, permettant ainsi leur diversification ([3]). Les autres filtres environnementaux identifiés correspondent au ratio C/N (0.5%), à la teneur en potassium (0.5%) et à la température annuelle moyenne (0.4%). Hormis la température, elles ont toutes un effet négatif sur la richesse taxonomique bactérienne, potentiellement car leur augmentation traduit une diminution de l'hétérogénéité des niches à micro échelle.

4.1.3 Structure des communautés bactériennes des sols

Articles [4-5], [9-10], [14].

Dans cette partie, je me focalise sur les variations de structure des communautés bactériennes des sols (diversité β). Les résultats présentés s'appuient sur la caractérisation des communautés bactériennes du sol par une technique d'empreinte moléculaire (ARISA, Ranjard *et al.*, 2001), les résultats s'appuyant sur les techniques de séquençage haut débit étant en cours de publication. L'IGS bactérien présente un polymorphisme taille qui permet de caractériser la structure des communautés bactériennes du sol au travers de l'abondance relative de chaque taille de bande à raison de 100 bandes par échantillon ([14]).

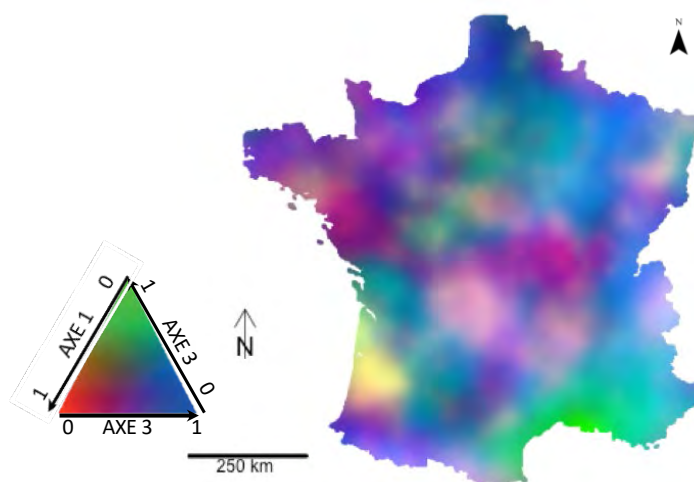


Figure 9. Cartographie de la structure des communautés bactériennes des sols à l'échelle du territoire national

La représentation des variations de structure des communautés bactériennes des sols est réalisée en interpolant les scores normalisés des sites sur les trois premiers axes d'une analyse NMDS. Les trois cartes sont ensuite superposées et un mélange de couleurs est réalisé selon la codification RVB. Le triangle de mélange des couleurs est proposé à gauche de la carte. Dans cette représentation, deux sites présentant des couleurs différentes ont des structures de communautés différentes. Cette carte a servi de base dans la rédaction de l'article [14] mais n'a pas été publiée.

La Figure 9 présente les variations de structure des communautés bactériennes des sols sur la base d'une ACP permettant d'avoir une représentation des différences relatives entre chaque site. Ainsi, sur la carte de la Figure 9, deux sites présentant des couleurs différentes ont des communautés bactériennes différentes. A l'échelle du territoire national, on observe des variations de structure de communautés entre régions : e.g. Landes, Languedoc-Roussillon, Massif Central, Bretagne et Nord-Est de la France. Ces variations sont structurées spatialement à grande échelle puisque le rayon des unités spatiales est d'au minimum 140 km de rayon [14]. La structure des communautés bactériennes est donc structurée spatialement à l'échelle du territoire national, conclusion qui est en accord avec la littérature tant à des échelles continentales ou de pays (Green & Bohannan, 2006; Martiny *et al.*, 2006; Fierer *et al.*, 2007; Powell *et al.*, 2015; Delgado - Baquerizo *et al.*, 2017; Thompson *et al.*, 2017; Delgado-Baquerizo *et al.*, 2018). Néanmoins, la variabilité locale de la structure des communautés bactériennes des sols est sans doute très forte au regard de l'effet pépite des variogrammes qui est d'au moins 57%.

L'analyse des variations de structure des communautés bactériennes a été réalisée à partir des données de fingerprint moléculaire et de séquençage haut débit sur la base de leur composition (présence/absence des différentes bandes/OTU)

dans quatre régions contrastées : Landes, Bretagne, Bourgogne, Sud-Est de la France ([9-10]). Cette analyse de la variance de la structure des communautés bactériennes des sols a été réalisée par une analyse canonique des redondances sur matrice de distance (db-RDA, Legendre & Anderson, 1999; Legendre *et al.*, 2009) en utilisant l'indice de Jaccard comme mesure de dissimilarité entre sites. Cette analyse a mis en œuvre trois groupes de variables explicatives : les caractéristiques physico-chimiques du sol, l'utilisation du sol et un groupe de descripteurs géographiques. Ces descripteurs géographiques correspondent à des PCNMs (Principle Coordinates of Neighbour Matrix ; Dray *et al.*, 2006) et ont la particularité d'être tous indépendants et de représenter une échelle spatiale pouvant être estimée au travers de leur semi-variogramme. La prise en compte de ces trois groupes de variables dans l'analyse a permis de tester l'importance relative des processus de sélection et de dispersion dans la structuration spatiale des communautés bactériennes des sols ; et d'identifier et de hiérarchiser les filtres environnementaux impliqués dans le processus de sélection environnementale.

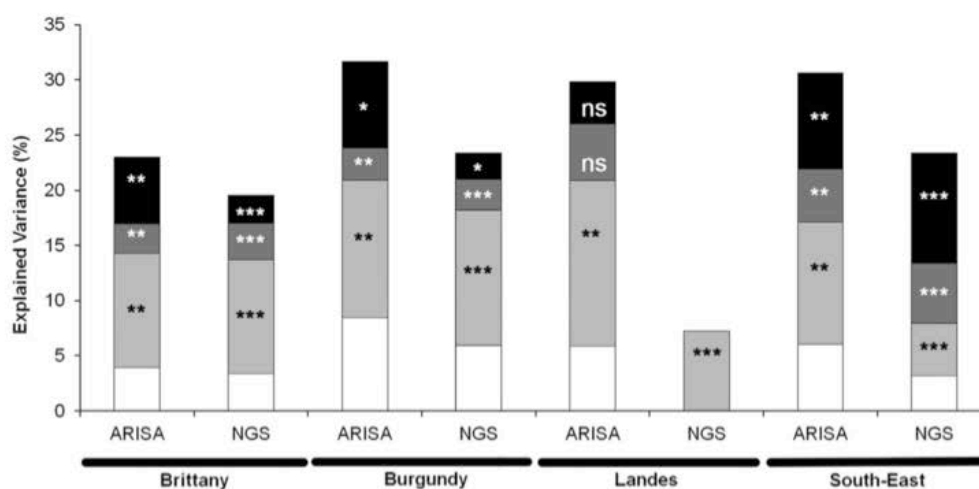


Figure 10. Partition de la variance de la structure des communautés bactériennes des sols

La structure des communautés bactériennes des sols a été caractérisées par une technique de fingerprint moléculaire (ARISA) et par une technique de séquençage haut débit (Pyroséquençage 454, Roche, notée NGS). Les groupes de variables sont représentés comme suit : caractéristiques physico-chimiques du sol (gris clair), utilisation du sol (gris foncé), descripteurs géographiques (noir), interactions entre caractéristiques physico-chimiques des sols et utilisation du sol (blanc). Les seuils de significativité sont les suivants : ns : $P > 0.05$; * : $P < 0.05$; ** : $P < 0.01$; *** : $P < 0.001$.

Quelle que soit la méthode utilisée pour caractériser les communautés bactériennes des sols à l'échelle des quatre régions, l'ensemble physico-chimie du sol et utilisation du sol explique la part la plus importante des variations de structure des

Chapitre 2

communautés bactériennes, ceci indépendamment de leurs interactions (Figure 10). Ceci traduit le fait que la sélection environnementale est sans doute le premier processus structurant les communautés bactériennes des sols. Les descripteurs spatiaux expliquent une part plus faible mais significative des variations de structure des communautés bactériennes des sols dans toutes les régions excepté les Landes. Ces derniers peuvent traduire des gradients environnementaux non mesurés ou bien des relations spatiales entre sites, *i.e.* des migrations d'un site A vers un site B. Ici, vu le grand nombre de variables environnementales prises en compte dans l'analyse, il apparaît peu probable que les descripteurs spatiaux correspondent uniquement à des gradients environnementaux non mesurés. Ils traduisent donc probablement une limite à la dispersion des communautés bactériennes du sol. Les processus stochastiques comme la dispersion semblent jouer un rôle significatif mais plus faible que la sélection environnementale. Ces observations sont en accord avec la littérature puisque le processus de sélection environnementale est systématiquement identifié comme structurant pour les communautés microbiennes des sols (Horner-Devine *et al.*, 2004; Drenovsky *et al.*, 2010; Griffiths *et al.*, 2011; Hanson *et al.*, 2012; Powell *et al.*, 2015). L'importance des limites à la dispersion est plus réduite et moins fréquemment observée (Hanson *et al.*, 2012) mais a tout de même été démontrée pour certains genres (Cho & Tiedje, 2000) ou pour la communauté totale (Powell *et al.*, 2016). Ici, les groupes de PCNMs représentant des structures spatiales à des échelles larges (80 à 120 km de rayon) et plus fines (40 à 65 km de rayon) expliquaient tous deux des parts significatives de la variance des communautés bactériennes des sols. Ceci suggère que la limite à la dispersion pour les microorganismes pourrait être effective à différentes échelles spatiales.

L'analyse fine des filtres environnementaux structurant les communautés bactériennes des sols a été réalisée sur les données d'empreinte moléculaire. Parmi les variables de physico-chimie des sols, chaque variable présente un effet marginal relativement faible étant donné le nombre important de variables explicatives sélectionnées. La hiérarchie des variables de physico-chimie des sols varie un peu entre les différentes régions. Néanmoins, en Bretagne, Bourgogne et dans le Sud-Est de la France, le pH est le premier filtre structurant les communautés bactériennes de sols. Il est suivi par des variables liées à la quantité et à la qualité des ressources trophiques dans les sols (teneur en azote total : N_{tot} ; C_{org} , ratio C/N)

et par la texture du sol (teneur en argiles et en limons) ; puis par la teneur en nutriments tels que le potassium ou le magnésium. En dernier, et uniquement en Bretagne, les précipitations annuelles moyennes constituent un filtre structurant les communautés bactériennes des sols. Contrairement aux trois autres régions, les communautés bactériennes de la région des Landes ne semblent structurées que par la qualité de la ressource trophique puisque seul le ratio C/N est significatif. Ceci peut s'expliquer par le caractère particulier de cette région qui présente une variabilité très faible en termes de types de sols (principalement des podzosols) ou d'utilisation du sol (principalement des couverts forestiers). Le rôle majeur du pH identifié ici est aujourd'hui bien démontré dans la littérature (Fierer & Jackson, 2006; Griffiths *et al.*, 2011, 2016; Hanson *et al.*, 2012; Terrat *et al.*, 2014, 2017). Le rôle prépondérant de l'utilisation du sol est aussi régulièrement identifié dans la littérature, en particulier lorsque des systèmes contrastés sont considérés (Drenovsky *et al.*, 2010; Dumbrell *et al.*, 2010). Il est moins prégnant lorsque les variations d'utilisation du sol sont plus graduelles (Delgado-Baquirezo *et al.*, 2017). Ces différences peuvent être liées à un effet direct de la diversité végétale épigée qui a été identifiée comme pouvant structurer les communautés microbiennes (bactéries : Thompson *et al.*, 2017 ; champignons : van der Heijden *et al.*, 1998; Waldrop & Firestone, 2006). Elles peuvent aussi être liées aux interactions entre utilisation du sol et caractéristiques physico-chimiques des sols, en particulier le pH, la teneur en C_{org} et le ratio C/N. Ces dernières constituent elles aussi des variables environnementales déterminantes dans la structuration des communautés bactériennes des sols même si elles apparaissent moins fréquemment significatives, notamment du fait de leur forte corrélation avec l'utilisation du sol.

4.1.4 Étude de la TAR

Articles [9] & [14].

La relation Aire-Taxon (TAR) traduit l'augmentation de la richesse taxonomique (S_A) avec la surface échantillonnée (A). Elle permet d'estimer le turnover des communautés (z) en évaluant le taux d'accumulation de nouvelles espèces en fonction de l'augmentation de l'aire d'échantillonnage à partir d'un point de richesse spécifique S_0 . Étudiée depuis le début de l'écologie quantitative, elle est souvent

représentée sous la forme d'une équation d'Arrhénius (Arrhenius 1921; Gleason 1922).

$$S_A = S_0 * A^z$$

Équation 1. Équation de la relation Aire-Taxon (TAR)

Avant le développement et le déploiement massif des techniques de séquençage haut débit, réaliser un dénombrement représentatif du nombre de taxons bactériens ou fongique dans les sols était hors de portée. Pour estimer le turnover des communautés microbiennes, on s'appuyait alors sur la décroissance de la similarité entre communautés avec l'augmentation de l'interdistance (Green et al., 2006, Harte et al., 2009) ; en faisant l'hypothèse que les communautés microbiennes ont un nombre d'individus "infini", ceci en regard du nombre d'individus chez les macroorganismes ; et que la valeur de z est stable à l'échelle considérée [10].

$$\chi_d = \chi_D * \left(\frac{d}{D}\right)^{-2z} \Leftrightarrow \log_{10}(\chi_d) = -2z * \log_{10}(d) + c$$

Équation 2. Équation de la Décroissance de Similarité en fonction de la distance (DDR)

χ_d est la similarité d'habitat entre deux sites distants de d, χ_D est la similarité d'habitat entre deux sites distants de D et z le turnover des communautés. c : constante

L'étude de la relation Aire-Taxons à l'échelle

$$\chi_d = \chi_D * d^D - 2z \Leftrightarrow \log_{10} \chi_d = -2z * \log_{10} d + c$$

Équation 2 a permis de mettre en évidence une relation significative entre la similarité génétique et la distance géographique (Figure 11).

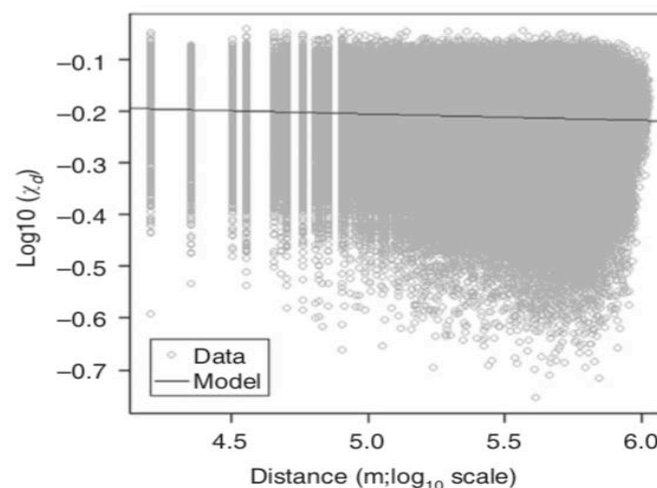


Figure 11. TAR à l'échelle de la France.

Cercles gris: Similarité génétique entre paires de sites (échelle logarithmique) en fonction de l'interdistance entre sites (m, échelle logarithmique) geographical distance (m); Ligne noire : modèle de régression linéaire pondérée de la similarité génétique en fonction de la distance géographique. Le

Chapitre 2

poinds de chaque point est égal au nombre de points dans la classe de distance. Equation du modèle ($P < 0.001$) : $\log_{10}(\text{genetic similarity}) = 0.0117 * \log_{10}(\text{geographical distance}) - 0.1464$.

Cette relation confirme que les communautés bactériennes des sols sont distribuées de manière hétérogène et structurée à l'échelle du territoire national. Ceci est en accord avec d'autres études à des échelles larges (Green *et al.*, 2004; Horner-Devine *et al.*, 2004; Green & Bohannan, 2006; Zhou *et al.*, 2006; Martiny *et al.*, 2006; Powell *et al.*, 2015). La valeur du turnover (z) est estimée à 0.006 (+/- $7 \cdot 10^{-4}$, $P < 0.001$), valeur qui est aussi en accord avec les études mettant en oeuvre les études méttant en oeuvre la même approche (Horner-Devine *et al.*, 2004; Fierer *et al.*, 2008, [15]). Néanmoins, elle est très inférieure aux nouvelles études de biogéographie microbienne mettant en oeuvre des techniques de séquençage haut débit (Powell *et al.*, 2016 ; [9]). Cette différence entre études s'explique principalement par l'approche choisie pour estimer la valeur du z et le type de données disponibles (Zinger *et al.*, 2014 ; [9]). En effet, les techniques d'empreintes moléculaires sont moins résolutive que les approches de séquençage massif (plusieurs espèces par bandes et polymorphisme de bandes au sein d'une même espèce). Elles saturent donc plus vite et aboutissent alors à une sous-estimation du turnover des communautés microbiennes [9] et le séquençage massif offre la possibilité de mieux estimer la valeur de z . Même si cette différence ne semble pas remettre en cause les conclusions entre études, les études basées sur les données de séquençage massif pourraient conduire à réexaminer l'importance relative des différents processus écologiques mis en évidence plus haut dans la structuration des communautés microbiennes des sols (Hanson *et al.*, 2012, [9]). Par ailleurs, les valeurs de z observées avec les méthodes de NGS sont du même ordre de grandeur que celles observées pour les macroorganismes (0.1 – 0.8 ; Horner-Devine *et al.*, 2004; Drakare *et al.*, 2006), contrairement à la relation positive classiquement observée en écologie entre la taille d'un organisme et la valeur du z (Hillebrand *et al.*, 2001; Drakare *et al.*, 2006). Ceci laisse supposer que le turnover des microorganismes du sol pourrait être, comme pour les macroorganismes, influencé par la diversité des habitats et leur configuration spatiale.

Dans ce cadre, j'ai testé la relation entre le turnover des communautés bactériennes des sols et le turnover et la configuration spatiale des habitats microbiens [14]. Les habitats microbiens ont été caractérisés par les coordonnées des sites dans une ACP mixte (méthode de Hill & Smith) incluant les caractéristiques

physico-chimiques des sols, leur utilisation et les caractéristiques climatiques. Sur la base de cette ACP, j'ai estimé le turnover des habitats microbiens calculant la similarité entre habitats microbiens à l'aide de l'Équation 3 et en transposant la DDR (

$$\chi_d = \chi_D * \left(\frac{d}{D}\right)^{-2z} \Leftrightarrow \log_{10}(\chi_d) = -2z * \log_{10}(d) + c$$

Équation 2) pour estimer le turnover des habitats microbiens (z_{Habitat}).

$$E_d = \left(1 - \frac{Eud_d}{Euc_{max}}\right) + 0.001$$

Équation 3. Similarité entre habitats microbiens

E_d est la similarité d'habitat entre deux sites. Euc_d est la distance euclidienne entre deux sites distants de d et Euc_{max} est la distance euclidienne maximale entre sites. On y ajoute 0.001 pour prendre en compte le cas où la distance euclidienne entre les deux sites est maximale.

Afin d'avoir une taille d'échantillon suffisante pour évaluer la relation entre le turnover des habitats microbiens (z_{habitat}) et le turnover des communautés bactériennes des sols (z_{bacteria}), j'ai utilisé une approche de fenêtre glissante centrée sur chaque site du RMQS (rayon de la fenêtre 140km). Le résultat est présenté en Figure 12.

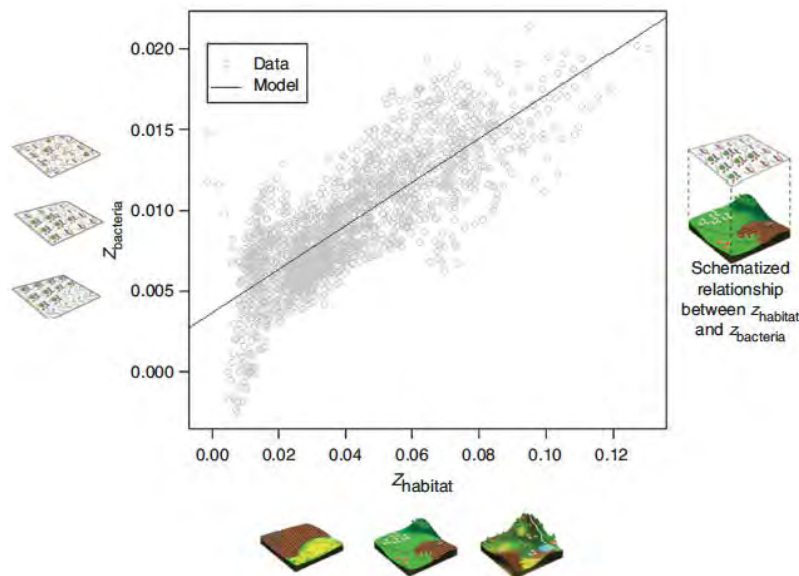


Figure 12. Relation entre les turnovers des communautés bactériennes du sol et de l'habitat microbien. Les ronds gris représentent les valeurs estimées pour chaque site du RMQS par la méthode de la fenêtre glissante décrite en [15], chaque site étant au centre d'une fenêtre de 140km de rayon. La ligne noire correspond au modèle linéaire de type II ajusté aux données : $Z_{bacteria} = 0.1350 * Z_{habitat} + 0.0036$, ($r^2 = 0.65$, $P < 0.001$). Les schémas sous l'axe des abscisses et à gauche de l'axe des ordonnées représentent la fragmentation de l'habitat microbien et la structure de communauté bactérienne

Chapitre 2

On observe que le turnover des communautés bactériennes est positivement et significativement relié à celui de l'habitat microbien ($r^2 = 0.65$). Ce résultat conduit à deux conclusions importantes. D'une part, plus le turnover des habitats microbiens augmente, i.e. plus les habitats sont diversifiés, plus le turnover des communautés bactériennes des sols est élevé. D'autre part, même si le turnover des habitats microbiens est nul, celui des communautés bactériennes des sols reste positif (ordonnée à l'origine du modèle linéaire). Ces observations ont permis de confirmer l'importance des processus de sélection environnementale et de dispersion dans la structuration spatiale des communautés bactériennes des sols. Par ailleurs, une analyse complémentaire a permis de tester l'importance de la configuration paysagère dans le déterminisme du turnover des communautés bactériennes des sols. Pour cela, au sein de chaque fenêtré, les sites ont été randomisés pour modifier aléatoirement la configuration spatiale des habitats sans modifier le lien entre communauté bactérienne du sol et habitat. Cette randomisation a été répétée 1000 fois par fenêtré. Ceci a permis de montrer que la relation observée en Figure 12 n'était pas due au hasard et que la valeur de la pente était bien dépendante de la configuration spatiale des habitats microbiens. Au final, le turnover des communautés bactériennes des sols est, comme celui des macroorganismes (MACARTHUR *et al.*, 1967), dépendant de la diversité des habitats et de leur configuration spatiale. Cette relation a récemment été testée sur un échantillonnage spatialisé en Ecosse et a abouti aux mêmes conclusions (Powell *et al.*, 2016).

4.2 Distribution spatiale et déterminisme de l'abondance, de la diversité et de la structure des communautés microbiennes à l'échelle du paysage agricole

Articles [7] & [8].

Les études à l'échelle du paysage agricole s'appuient sur la zone atelier de Féney. Localisé à 15km de Dijon en Côte d'Or (Région Bourgogne-Franche-Comté), il couvre 1200 ha dont surfaces agricoles (900ha) et de forêts (200ha). Le paysage de Féney compte 278 points de mesure répartis suivant une grille systématique de 215m de côté. Cette grille est représentative de la variabilité des sols et de la diversité des pratiques agricoles mises en œuvre (Figure 13). Sur ce dernier point, le paysage de Féney est particulièrement bien documenté puisque les pratiques agricoles sur chaque parcelle y ont été inventoriées sur les 12 dernières années. Je les ai développées en particulier dans le cadre de la thèse de Florentin Constancias, thèse que j'ai co-encadrée avec Lionel Ranjard de 2011 à 2015.

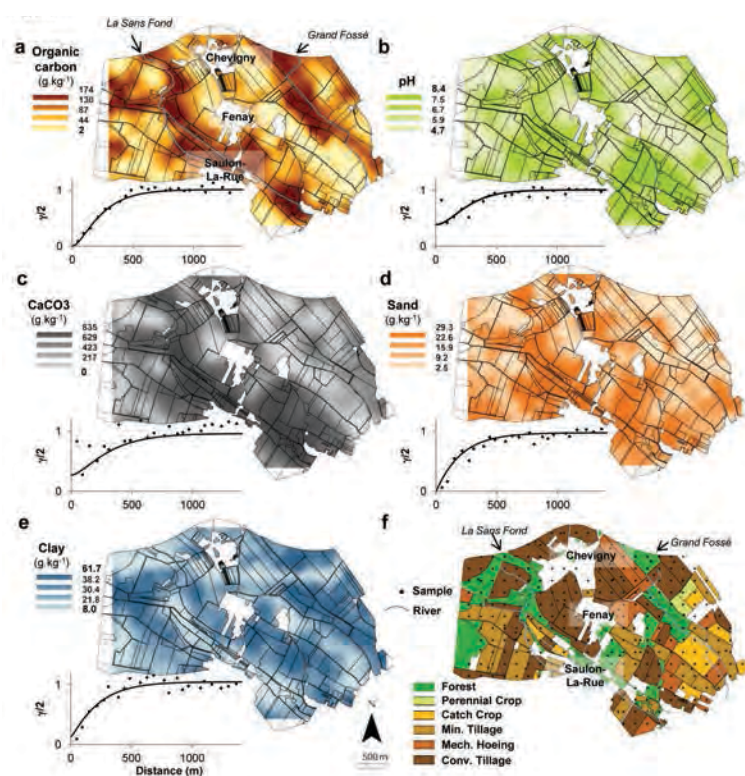


Figure 13. Variabilité des caractéristiques des sols du paysage agricole de Féney.

(a-e) Carte de la teneur en Carbone Organique, du pH, de la teneur en Carbonates, de la teneur en sables et de la teneur en argiles ; respectivement ; (f) Carte de l'utilisation des sols au regard des classes de pratiques agricoles : Forest : Forêts, Perennial Crop : Cultures pérennes, Catch Crop : rotations incluant des cultures intermédiaires pièges à nitrates (CIPAN), Min. Tillage : Cultures implantées par des méthodes de travail du sol réduit sans CIPAN, Mech. Hoeing : Cultures implantées après un décompactage sans CIPAN, Conv. Tillage : Cultures implantées après un labour sans CIPAN. Les points noirs correspondent aux sites d'échantillonnage.

4.2.1 Abondance des communautés microbiennes des sols

Article [8].

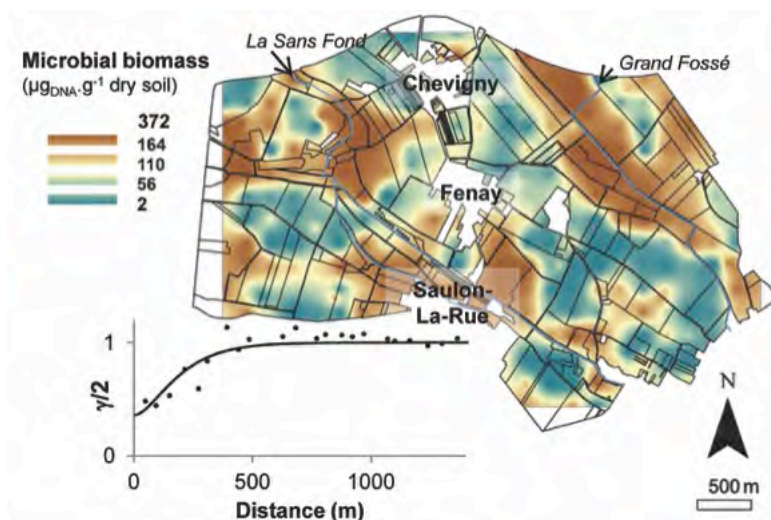


Figure 14. Cartographie de la biomasse moléculaire microbienne des sols à l'échelle du paysage de Féney.

Les noms en gras correspondent aux noms des communes incluent dans le paysage de Féney et les noms en italique correspondent aux deux cours d'eau traversant le paysage. En dessous de la carte, le variogramme utilisé pour réaliser l'interpolation est proposé. L'article associé est référencé sous le numéro [8].

A l'échelle d'un paysage agricole, l'abondance des communautés microbiennes varie entre 2 et 380 $\mu\text{g}_{\text{ADN}}\cdot\text{g}_{\text{sol}}^{-1}$ (Figure 14). Cette forte gamme de variation est du même ordre de grandeur que celles observées dans d'autres études à des échelles plus fines allant du bloc de sol (Nunan *et al.*, 2003), à la région (Berner *et al.*, 2011). Ces variations de biomasse moléculaire microbienne du sol sont distribuées de manière hétérogène et structurée suivant des unités spatiales de d'environ 515m de rayon. Même si l'échelle considérée est plus fine que celle du territoire national, il subsiste là encore une forte variabilité locale comme le suggère le fort effet pépite du variogramme (36%). Ceci est en accord avec une étude récente montrant que les paramètres microbiens d'un sol peuvent varier à l'échelle de quelques centimètres (O'Brien *et al.*, 2016). L'étude des déterminants de ces variations a permis de mettre en évidence le rôle prépondérant de la teneur en C_{org} (10.1%), de l'utilisation du sol et des pratiques agricoles avec un effet négatif des actions de travail du sol (17.3%). Les autres variables environnementales identifiées étaient le ratio C/N (1.5%), la teneur en argiles du sol (0.5%) et en carbonates (0.5%). Ces filtres sont en accord avec ceux identifiés plus haut à l'échelle du territoire national mais est aussi en accord avec la littérature qui met en avant l'importance quantitative et qualitative de

la matière organique pour la croissance microbienne dans les sols (Leckie *et al.*, 2004; De Boer *et al.*, 2005). Au regard des échelles plus larges, la faible influence de la teneur en argiles du sol peut s'expliquer par la faible variabilité texturale des sols (principalement argilo-limoneux ou limono-argileux). L'effet de l'utilisation du sol se décline ici à l'échelle des pratiques agricoles. Une classification ascendante hiérarchique des pratiques agricoles a permis de regrouper les parcelles suivant un gradient de travail du sol : Forêts (pas de travail du sol), Cultures pérennes (prairies et petits fruits ; travail du sol réduit), Grandes cultures avec cultures intermédiaires pièges à nitrates (CIPAN, implantation en travail du sol réduit), Grandes cultures sans CIPAN (implantation en travail du sol réduit), Cultures implantées après un décompactage sans CIPAN, Cultures implantées après un labour profond sans CIPAN. La prise en compte de ces différentes catégories a permis de mettre en évidence que le travail du sol induisait une diminution de la biomasse moléculaire microbienne. Ceci peut s'expliquer par une modification de l'accessibilité à la ressource carbonée. En effet, en déstructurant les agrégats, le travail du sol augmente l'accessibilité à la ressource carbonée. Ceci favorise des taxons bactériens dégradant des matières labiles et ayant une dynamique de population très forte avec une croissance très forte tant que la ressource est disponible qui s'arrête dès l'épuisement de celle-ci (stratégies r). Ceci conduit à terme à une réduction de la biomasse microbienne ([15-16]). A contrario, des systèmes sans travail du sol (prairies et forêts) favorisent des microorganismes consommant des matières organiques plus stables, dont à croissance plus lente mais dont la dynamique de population est aussi plus stable à long terme (stratégies K).

4.2.2 Richesse taxonomique des communautés bactériennes des sols

Article [8].

La Figure 15 présente la distribution spatiale de la richesse bactérienne à l'échelle du paysage de Fénay. La gamme de variation est assez large puisqu'elle varie de 850 à 1761 OTU par échantillon de sol (95% de similarité, profondeur de séquençage : 10800 séquences par échantillon), ce qui est en accord avec la bibliographie puisque des études comparables en termes de profondeur de séquençage ont montré une richesse bactérienne variant de 600 à 2750 OTU par

échantillon de sol tant à des échelles locale (Veach *et al.*, 2015; Hartman *et al.*, 2018; Tian *et al.*, 2018; Wang *et al.*, 2018).

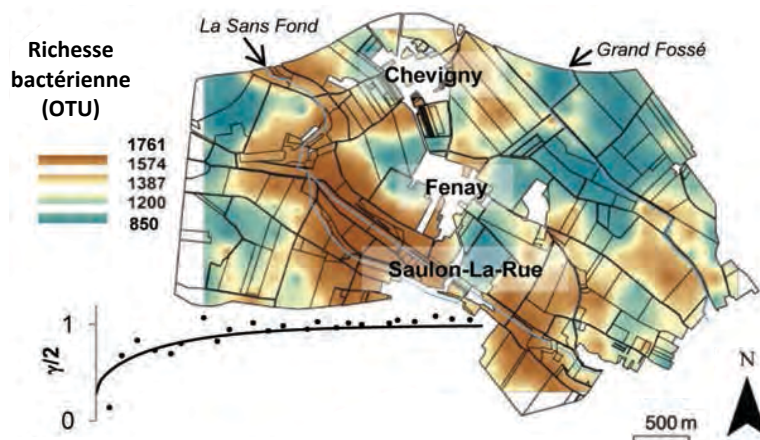


Figure 15. Cartographie de la richesse bactérienne des sols à l'échelle du paysage agricole de Fénay Les noms en gras correspondent aux noms des communes incluent dans le paysage de Fénay et les noms en italique correspondent aux deux cours d'eau traversant le paysage. Le variogramme sur lequel s'appuie l'interpolation est présenté sous la carte. L'article associé est référencé sous le numéro [8].

Le variogramme traduit une relation significative entre la semi-variance de la richesse bactérienne des sols et l'interdistance entre les sites de prélèvements, indiquant qu'elle est distribuée de manière hétérogène et structurée à l'échelle du paysage agricole. Cette structuration se traduit par des unités spatiales de 810m de rayon, même si la variabilité locale, en particulier intraparcellaire, reste forte puisque l'effet pépite est d'environ 30%.

Pour identifier les filtres environnementaux déterminant le niveau de richesse bactérienne à l'échelle du paysage agricole de Fénay, les mêmes méthodologies ont été mises en œuvre qu'à l'échelle du territoire national. Les caractéristiques physico-chimiques des sols expliquent la plus grande part de variance de la richesse bactérienne (environ 45%), devant les pratiques agricoles (3.7%). Même si les caractéristiques physico-chimiques des sols du paysage de Fénay présentent de fortes interactions les unes avec les autres, le pH du sol (5.9%), la teneur en Argiles (6.1%) et la teneur en carbonates (2.1%) ressortent comme les filtres les plus importants, ceci avec les pratiques agricoles. Comme à l'échelle du territoire national, l'effet positif du pH, et négatif de la teneur en argiles peut s'expliquer par la modification des conditions réactionnelles et la réduction de l'hétérogénéité environnementale à micro-échelle réduisant le nombre de niches disponibles. Par ailleurs, une analyse plus fine des pratiques agricoles montre que le travail du sol

tend à augmenter la richesse bactérienne. Ceci s'explique bien par la théorie des perturbations intermédiaires. En déstructurant les agrégats, le travail du sol favorise la création de nouvelles niches écologiques tout en perturbant peu pour les bactéries du fait de leur petite taille et de leur relative protection au sein des micro-agrégats ([12], [15]) ; contrairement aux champignons qui sont très affectés par le travail du sol d'après Lienhard et al. (2013). Néanmoins, cette augmentation de richesse bactérienne n'est pas forcément positive puisque l'on observe une augmentation des genres appartenant aux stratégies *r*.

4.2.3 Structure des communautés bactériennes des sols

Articles [7].

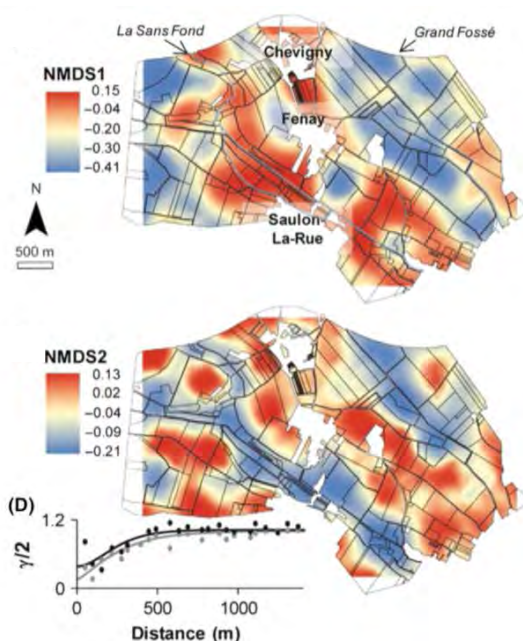


Figure 16. Cartographie de la structure des communautés bactériennes des sols à l'échelle du paysage agricole de Féney.

La représentation des variations de structure des communautés bactériennes des sols est réalisée en interpolant les scores normalisés des sites sur les deux premiers axes d'une analyse NMDS (NMDS1 et NMDS2). Les deux cartes sont ici juxtaposées. Pour chaque carte, des couleurs différentes entre sites traduisent des différences en termes de structure des communautés bactériennes. Les noms en gras correspondent aux noms des communes incluent dans le paysage de Féney et les noms en italique correspondent aux deux cours d'eau traversant le paysage. Les variogrammes sur lesquels s'appuient les interpolations sont présentés sous la carte. L'article associé est référencé sous le numéro [7].

La Figure 16 présente les variations de structure des communautés bactériennes des sols sur la base d'une analyse canonique (NMDS) en s'appuyant sur la distance

Chapitre 2

"weighted-Unifrac" pour mesurer la dissimilarité entre les échantillons. Elle s'appuie pour cela sur des données de séquençage massif de l'ADN extrait du sol après amplification ciblée de l'ADNr 16S (F479 (5'-CAGCMGCYGCNGTAANAC-3') et R888 (5'-CCGYCAATTCMTTTRAGT-3')). En maximisant les différences entre les échantillons, elle permet d'avoir une représentation des différences relatives entre chaque site. Ainsi, sur les cartes de la Figure 16, deux sites présentant des couleurs différentes ont des communautés bactériennes différentes. A l'échelle du paysage agricole de Féney, la structure des communautés bactériennes varie de manière non aléatoire et est structurée spatialement avec des unités spatiales dont le rayon est de 741m pour l'axe NMDS1, 524m pour l'axe NMDS2. Il est possible de distinguer des zones très contrastées : des zones bleues sur les deux cartes à l'est et à l'ouest de la zone correspondant à des zones forestières ; et une zone rouge sur la carte de l'axe NMDS1 localisée autour de la Sans-fond, rivière principale de la zone. Cette structuration spatiale est en accord avec la littérature à des échelles "équivalentes" à une échelle paysagère s'entendant ici comme l'étude de plusieurs écosystèmes dans une même zone géographique (Jia *et al.*, 2006; Drenovsky *et al.*, 2010) ; et à l'échelle de la parcelle (Bell *et al.*, 2005; O'Brien *et al.*, 2016).

L'analyse de la variance de la structure des communautés bactériennes de sols met en évidence que la physico-chimie du sol, l'utilisation du sol et leurs interactions expliquent respectivement 24.0%, 6.8% et 39.9% des variations de la structure des communautés bactériennes des sols. Les descripteurs spatiaux expliquent quant à eux 2.7% des variations de la structure des communautés bactériennes des sols. Ainsi, la sélection environnementale semble être le premier processus structurant les communautés bactériennes des sols à l'échelle du paysage de Féney. Néanmoins, la dispersion semble aussi importante au regard de l'effet significatif des descripteurs spatiaux. Cette hypothèse est renforcée par la modélisation de l'abondance relative des différents taxons identifiés sur la zone de Féney qui montre que certains taxons sont surreprésentés dans des sites où leur abondance est estimée à des valeurs plus faibles au regard de leurs caractéristiques environnementales. Ceci laisse supposer que ces taxons sont soumis à des effets de masse maintenant des tailles de population élevées en dépit de conditions environnementales peu favorables ([7]). L'analyse plus détaillée des filtres environnementaux supportant le processus de sélection montre que la hiérarchie des filtres à l'échelle du paysage est : pH du sol (17.9%), utilisation du sol & pratiques agricoles (6.8%), teneur en carbonates (4.2%)

& C_{org} (0.6%). Cette hiérarchie est en accord avec la littérature (Drenovsky et al., 2010 ; Dumbrell et al., 2010) et les résultats observés à l'échelle du territoire national.

4.3 Synthèse

L'abondance des microorganismes du sol, la richesse bactérienne (diversité α) et la structure des communautés bactériennes (diversité β) des sols sont toutes distribuées de manière hétérogène et structurée spatialement à différentes échelles : le territoire national et le paysage agricole. Cette conclusion peut aussi être étendue à d'autres communautés microbiennes, en particulier les champignons ([10], Green et al., 2004; Peay et al., 2007; Powell et al., 2015). Tout n'est donc sans doute pas partout contrairement au postulat historique de Baas Becking (1934). Le turnover significatif de la diversité bactérienne, comme de la diversité de champignons, dont les valeurs sont proches de celles des macroorganismes lorsque l'exhaustivité de l'inventaire taxonomique augmente (avec l'utilisation des techniques de séquençage massif) montre que certaines théories développées en écologie des communautés des macroorganismes seraient transposables en écologie microbienne. Ainsi, tant à l'échelle du territoire national qu'à celle du paysage de Fénay, on observe que les communautés bactériennes sont structurées par au moins deux processus non-exclusifs : la sélection environnementale et la dispersion (Peay et al., 2007 ; Powell et al., 2016). La sélection environnementale met en jeu les caractéristiques physico-chimiques du sol et l'utilisation du sol ; mais aussi l'hétérogénéité environnementale en termes de diversité et de configuration spatiale des habitats paysagère. Examiner en détail les filtres impliqués dans la sélection environnementale montre que, globalement, les mêmes variables sont identifiées à l'échelle du territoire national et à l'échelle du paysage : pH, texture du sol, occupation du sol/pratiques agricoles. Cette homogénéité entre échelles montre qu'il est sans doute possible de transposer les concepts et les conclusions d'une échelle à l'autre (Harte et al., 2009). Ceci offre la possibilité de développer des outils sur la base d'informations acquises à une échelle donnée et de les déployer à une échelle spatiale différente.

Néanmoins, la compréhension de la diversité β varie fortement d'une échelle à l'autre et une part non négligeable de la variance de la structure des communautés bactériennes des sols reste inexpliquée. Ceci met en avant l'importance de

Chapitre 2

considérer d'autres filtres pouvant structurer les communautés microbiennes des sols telles que les interactions biotiques. En effet, une communauté n'est pas un simple assemblage de taxons indépendants mais plutôt une cohorte de taxons en interactions. Aujourd'hui, le déploiement du séquençage haut débit sur des échantillonnages de grande ampleur permet de mettre en œuvre des analyses de réseau de co-occurrence. Elles apportent de nouvelles connaissances en écologie microbienne en permettant de caractériser les relations entre les différents taxons microbiens en termes d'intensité et de type : synergie ou antagonisme ([4]). Ces approches tendent à se développer de plus en plus et montrent toute la complexité des interactions entre taxons microbiens dans le sol, interactions qui peuvent être sous la dépendance de facteurs environnementaux tels que la physico-chimie du sol (Delgado-Baquirezo et al., 2018), de l'occupation du sol ou des pratiques agricoles (O'Brien et al., 2016, Hartman et al., 2018).

Suite à tous les travaux engagés sur le RMQS depuis plus de 10 ans, un ouvrage de synthèse est en cours de publication. Cet ouvrage naturaliste est intitulé « Atlas français des bactéries du sol » et a été réalisé dans le cadre du post-doctorat de B. Karimi (Direction L. Ranjard et N. Chemidlin Prévost-Bouré ; financement ADEME, INRA, AgroSup Dijon). Il dresse un état des lieux de la biodiversité microbienne des sols français, en particulier au travers de trois ensembles de fiches. Le premier décrit les caractéristiques globales des communautés microbiennes des sols (abondance, diversité, composition et interactions). Le second décrit pour chaque phyla bactérien et d'archées : son aire de répartition en France, son autécologie, et ses interactions avec les autres phyla au travers de la bactériosociologie, ceci par analogie à la phytosociologie. Le troisième identifie des habitats microbiens et en décrit pour la première fois la signature microbienne et leur bactériosociologie. A destination des étudiants, des enseignants, des chercheurs, des acteurs socio-professionnels, des gestionnaires de la biodiversité, et du grand public, il constitue à la fois une ressource pédagogique, une source d'informations pour la gestion pratique de la biodiversité microbienne et un outil de vulgarisation.

5 Développement et transfert d'outils de diagnostic de la qualité microbiologique des sols en appui du développement agricole

Cette partie s'appuie en particulier sur les articles référencés sous les numéros [3], [5] & [17] et sur le projet de recherche participative AgrInnov (CASDAR IP 2012-2015, Chef de File : Observatoire Français des Sols Vivants ; Coordination scientifique : L. Ranjard). Elle constitue la part finalisée de mes activités de recherche.

Cette partie présente mes travaux de recherche finalisée qui se décomposent en deux temps. Tout d'abord, je participe au développement d'outils de diagnostic d'impact des pratiques agricoles puis je m'implique dans leur déploiement et leur mise en œuvre directement auprès des acteurs du monde agricole dans le cadre de réseaux de recherche participative.

5.1 Développement d'un outil de diagnostic de la qualité microbiologique des sols

Articles [3], [5] & [17].

Les études d'écologie spatiale des communautés microbiennes des sols, en particulier les travaux menés sur le RMQS et le paysage agricole de Fénay, mettent en évidence la sensibilité des communautés microbiennes (abondance et diversité) aux pratiques agricoles. Le RMQS étant représentatif de la diversité des sols français et de leur utilisation fait qu'il constitue aujourd'hui référentiel unique en son genre de l'état microbiologique des sols de France. Les études de standardisation technique ont démontré la robustesse et la reproductibilité des mesures de biomasse moléculaire microbienne et de diversité des bactéries et des champignons du sol (Plassart *et al.*, 2012; Terrat *et al.*, 2012). Ainsi, les communautés microbiennes des sols constituent des bioindicateurs d'impact des pratiques agricoles aujourd'hui reconnus nationalement^{1,2} ([25 - 26]).

A partir du référentiel RMQS, un outil de diagnostic de l'état microbiologique des sols a été construit (Post-Doctorat de W. Horrigue, dir. : L. Ranjard). Il s'appuie sur

¹ <http://indicateurs-biodiversite.naturefrance.fr/indicateurs/evolution-de-la-biomasse-microbienne-des-sols-en-metropole>

² <http://indicateurs-biodiversite.naturefrance.fr/indicateurs/evolution-de-la-biodiversite-bacterienne-des-sols>

Chapitre 2

l'évaluation de quatre indicateurs microbiens. Le premier est la biomasse moléculaire microbienne qui renseigne sur l'abondance des microorganismes du sol et permet d'évaluer un niveau d'activité microbiologique. Le second est le ratio champignons/bactéries (ratio entre la densité des champignons et la densité des bactéries) qui permet d'évaluer si la décomposition de la matière organique est préférentiellement médiée par les bactéries ou les champignons du sol. Les deux derniers correspondent à la richesse bactérienne et la richesse en champignons qui permettent d'évaluer le niveau d'assurance écologique (Loreau et al., 2000) associé aux microorganismes du sol puisque la diversité de ces est liée à la diversité, l'intensité et la redondance des fonctions biologiques du sol (Philippot *et al.*, 2013; Fierer *et al.*, 2014; Tardy *et al.*, 2014). Évaluer ces quatre indicateurs nécessite de définir des valeurs caractéristiques. Dans le cas du ratio Champignons/Bactéries, la mesure réalisée par PCR quantitative en temps réel ([17]) est comparée à des valeurs caractéristiques issues de la littérature : un ratio compris entre 1% et 5% indique qu'il n'y a pas de déséquilibre entre bactéries et champignons dans l'échantillon (Figure 17).



Figure 17. Diagramme de diagnostic du ratio champignons/bactéries.

Les valeurs caractéristiques sont indiquées à 1% et 5%. La flèche verte positionne la valeur mesurée. Les couleurs indiquent le diagnostic : vert : bon équilibre microbien ; jaune/orange : léger déséquilibre bactérien (ratio <1%) ou fongique (ratio > 5%) ; rouge : déséquilibre bactérien (ratio <1%) ou fongique (ratio > 5%).

La définition à la parcelle des valeurs caractéristiques de la biomasse moléculaire microbienne, de la richesse bactérienne et de la richesse en champignons met en œuvre trois modèles prédictifs et des résultats expérimentaux. Les modèles permettent d'estimer une valeur de référence (VR) de biomasse moléculaire microbienne, de richesse bactérienne ou de richesse en champignon à partir de la localisation géographique et des caractéristiques physico-chimiques du sol, ceci indépendamment des pratiques agricoles mises en œuvre ([3], [5]). L'équation générale des modèles est décrite en Équation 4.

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i=1}^n \beta_{iii} X_i^3 + \sum_{i=1}^{n-2} \sum_{j=i+1}^{n-1} \sum_{k=j+1}^n \beta_{ijk} X_j^i X_k + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j^2 + \sum_{i=1}^{n-2} \sum_{j=i+1}^{n-1} \sum_{k=i+j}^n \beta_{ijj} X_i X_j X_k + \dots + \varepsilon$$

Équation 4. Forme générale des modèles de diagnostic microbiologique du sol

Y est la variable à estimer, n est le nombre de variables explicatives sélectionnées, X_i est la $i^{\text{ème}}$ variable explicative, $i \in [1:n]$, β_i , β_{ij} et β_{ijk} étant les paramètres du modèle.

Chaque modèle a été construit pour respecter le principe de parcimonie tout en ayant une très bonne capacité prédictive. Les variables colinéaires ont été éliminées sur la base de leur corrélation ($|r| > 0.7$) et de leur facteur d'inflation de la variance (VIF >4). Le $R^2_{\text{ajusté}}$ et le BIC (Bayesian Information Criterion) ont permis d'ajuster la forme des modèles (*i.e.* le degré du polynôme) et une étape de cross-validation des modèles a été réalisée sur un sous-échantillon du RMQS indépendant du jeu d'apprentissage mais ayant la même distribution que le référentiel. *In fine*, chacun des trois modèles implique un jeu de variables environnementales qui lui est propre. Par exemple, le modèle de biomasse moléculaire microbienne implique le C_{org} , le pH, le taux d'argiles et l'altitude (en tant que proxy des précipitations annuelles moyennes) alors que le modèle de richesse bactérienne implique le pH, le taux d'argiles, le ratio C:N et la longitude (en tant que proxy du climat et de l'humidité du sol). Ils ne masquent pas l'effet de l'utilisation du sol ([3], [5]). L'erreur associée à la VR a été estimée expérimentalement à +/-20% dans le cadre de la thèse d'E. Bourgeois (encadrement PA Maron, co-encadrement : L. Ranjard ;Figure 18).

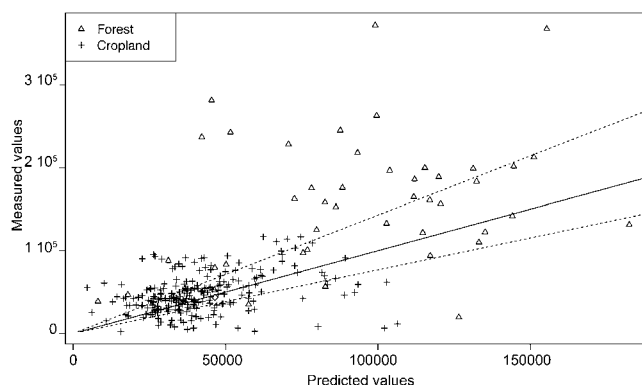


Figure 18. Relation entre la biomasse microbienne moléculaire prédite par le modèle et la biomasse moléculaire observée sur l'échantillonnage de Fény

Les valeurs prédites sont issues de l'utilisation du modèle présenté en équation 3. La ligne pleine correspond à la droite $y=x$; les lignes en pointillés correspondent à l'incertitude de mesure de la biomasse moléculaire microbienne ($\pm 20\%$). Les croix noires correspondent aux parcelles en grandes cultures et les triangles aux parcelles forestières.

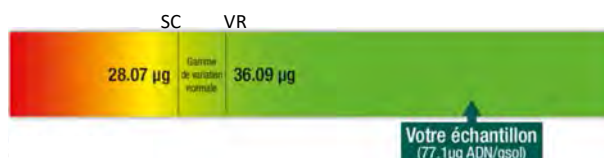


Figure 19. Exemple de diagramme de diagnostic s'appuyant sur les valeurs caractéristiques issues des modèles prédictifs.
 Les valeurs caractéristiques sont indiquées par VR (valeur de référence) et SC (seuil critique, $0.7 \times VR$). La flèche verte positionne la valeur mesurée. Les couleurs indiquent le diagnostic : vert : bon état ; jaune/orange : état à surveiller ; rouge : état à améliorer.

Par ailleurs, les travaux expérimentaux d'E Bourgeois, de V Tardy (Thèses de Doctorat, encadrement PA Maron & L Ranjard) et de PA Maron ont permis de mettre en évidence qu'une réduction de 30% de la biomasse moléculaire microbienne et des richesses bactérienne et de champignons induisait une modification significative du fonctionnement microbiologique du sol. Sur cette base, un seuil critique (SC) a été défini pour la biomasse moléculaire microbienne et les richesses bactérienne et de champignon avec $SC = 0.7 \times VR$. La comparaison de la valeur observée à ces valeurs caractéristiques permet d'évaluer l'effet améliorant/dépréciant des pratiques considérées (Figure 19). Le diagnostic microbiologique de la parcelle est ensuite réalisé en agrégeant les résultats des quatre indicateurs, chaque indicateur ayant un poids égal. Cette intégration se fait sous la forme d'une fiche de synthèse et permet alors de réaliser un diagnostic de l'impact des pratiques agricoles mises en œuvre sur la composante microbiologique du sol de la parcelle (Figure 20). Comme nous le verrons dans la suite, ce diagnostic microbiologique peut être intégré dans une évaluation plus large des pratiques agricoles/systèmes de culture.

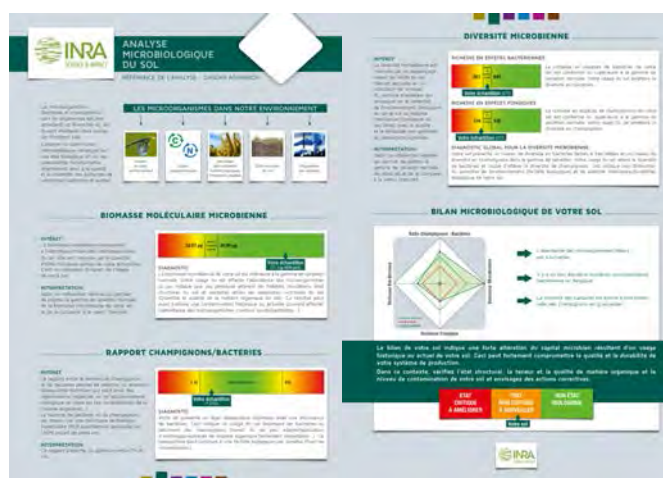


Figure 20. Fiche de diagnostic microbiologique du sol d'une parcelle agricole.
 Les encarts correspondent respectivement à : un descriptif général, l'évaluation de l'indicateur biomasse moléculaire microbienne, l'évaluation de l'indicateur ratio champignons/bactéries, l'évaluation des indicateurs de diversité microbienne (richesse bactérienne et en champignons) ; et à un bilan microbiologique synthétisé dans le dernier diagramme.

5.2 Transfert des outils de diagnostic de la qualité microbiologique des sols

Mes activités de recherche participatives s'appuient quant à elle sur des réseaux d'agriculteurs tels que celui créé dans le cadre du programme AgrInnov (Casdar 2012-2015, coord . L. Ranjard, et article référencé sous le numéro [29]).

La vision de l'agrosystème a évolué vers celle d'un agroécosystème hébergeant une biodiversité essentielle pour le fonctionnement des agroécosystèmes (projet Agroécologique pour la France, plan Ecophyto 1 et 2, programme fermes 30 000). Évaluer l'impact des pratiques sur la biodiversité [microbienne] des sols est aujourd'hui un enjeu important en agriculture pour maintenir/améliorer ce fonctionnement biologique. Les outils de diagnostic microbiologique adossés à des référentiels d'interprétation ([3], [5]) viennent répondre à ce besoin mais nécessitent d'être déployés à grande échelle pour identifier rapidement les pratiques permettant de maintenir le patrimoine biologique et ses fonctions supportant la fertilité biologique des sols. Les approches de recherche participative impliquant directement les agriculteurs offrent cette possibilité d'expérimentation massive, en complément des systèmes expérimentaux des instituts de recherche ou techniques, mais nécessitent un cadre théorique et technique pour être menées à bien.

Pour cela, je me suis impliqué dans le projet AgrInnov (CASDAR-IP 2012-2015, chef de File : Observatoire Français des Sols Vivants ; coordination scientifique : L. Ranjard, INRA ; collaboration AgroSup Dijon, ESA Angers, ISARA Lyon, IFV, ITAB, CA49) avec pour objectifs de : 1) Former les professionnels du monde agricole à la biologie des sols ; 2) Démontrer l'opérationnalité des indicateurs biologiques pour l'évaluation des pratiques agricoles directement à la ferme ou au domaine. Dans le cadre du projet AgrInnov, des formations et un tableau de bord d'indicateurs biologiques et agronomiques ont été co-construits avec les acteurs du monde agricole. Les indicateurs biologiques intégrés dans ce tableau de bord ont été choisis au regard de leur importance pour les acteurs du monde agricole et les formations ont été construites et évaluées pour maximiser l'autonomie des acteurs dans la mise en œuvre et l'interprétation du tableau de bord. Les indicateurs sélectionnés sont les indicateurs microbiens, les lombriciens et la nématofaune. Ils sont associés à trois indicateurs "agronomiques" : les analyses de terre, une mesure de l'état structural du sol (test bêche), une mesure de la dégradation de la matière organique

Chapitre 2

(LEVAbag^{MD}). Les formations étaient constituées de deux sessions à 6 mois d'intervalle : une session de formation théorique et pratique sur la biologie des sols et la mise en œuvre du tableau de bord ; et une restitution des résultats en groupe et individuelle.

Une fois construits, les formations et le tableau de bord d'indicateurs ont été déployés sur un réseau de recherche participative constitué de 125 fermes et 123 de domaines viticoles, réseau entièrement constitué dans le cadre du projet, les agriculteurs et les viticulteurs étant regroupés en fonction de leur proximité géographique dans des groupes de 12 à 16 personnes (Figure 21). Chaque groupe était suivi par un conseiller de chambre d'agriculture. Pour ma part, j'étais en charge du quart nord-est de la France, ce qui représentait 7 groupes de formation et 103 agriculteurs & viticulteurs.

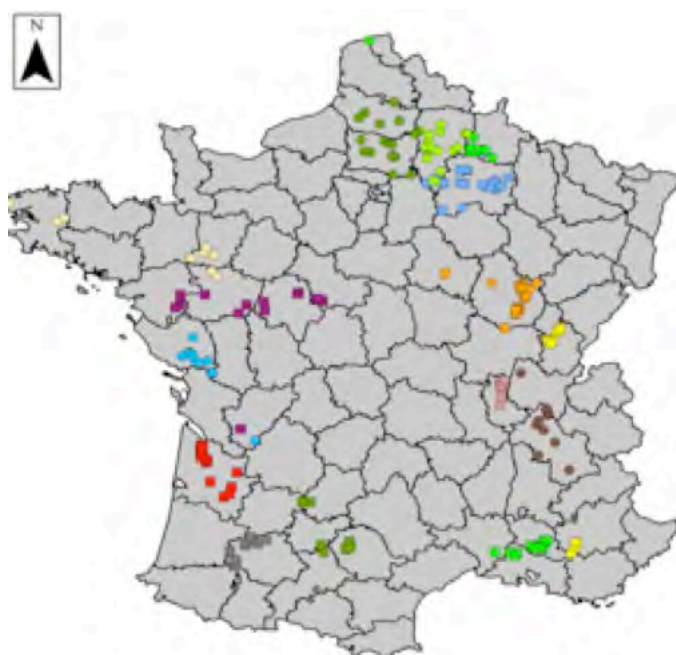


Figure 21. Localisation des fermes et des domaines viticoles du réseau du programme AgrInnov. Chaque couleur correspond à un groupe d'agriculteurs/de viticulteurs. Les fermes sont représentées par des ronds et les domaines viticoles par des carrés.

Ce programme constitue une réelle approche de recherche participative dans la mesure où les agriculteurs et les viticulteurs formés devaient mettre en œuvre le tableau de bord en autonomie. La démarche de co-construction et de mise en œuvre par des acteurs de la recherche s'est avérée efficace puisque 97% des personnes formées ont mis en œuvre le tableau de bord en fin de projet. Ceci démontre aussi l'opérationnalité des outils de diagnostic, en particulier des outils de diagnostic

microbiologique des sols. Par ailleurs, en association avec les acteurs du monde agricole, le programme AgrInnov a permis de construire deux indicateurs de synthèse afin d'agrégier les informations issues des différents indicateurs du tableau de bord. Après scoring, un indicateur de patrimoine biologique/assurance écologique et un indicateur de fertilité biologique ont été construits (Figure 22).

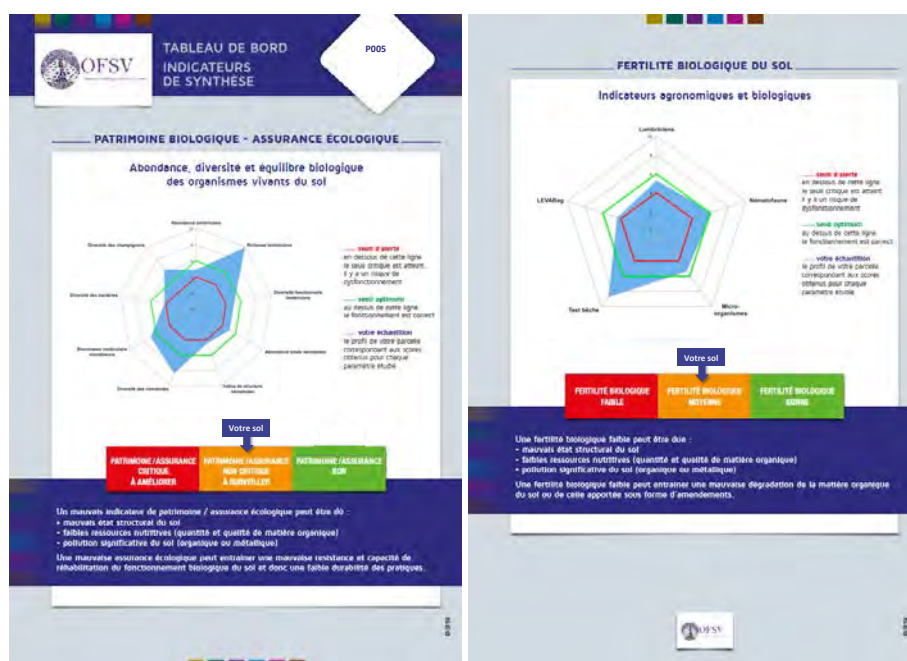


Figure 22. Indicateurs de synthèse du tableau de bord d'indicateurs du programme AgrInnov

La page de gauche correspond à l'indicateur de patrimoine biologique/assurance écologique. Il regroupe l'abondance, la richesse et la diversité fonctionnelle des lombriciens ; l'abondance, l'indice de structure et la diversité des nématodes ; et la biomasse moléculaire microbienne et la richesse bactérienne et en champignons. La page de droite correspond à l'indicateur de fertilité biologique. Il regroupe le test bêche, le LevaBag^{MD}, et une moyenne des scores pour les lombriciens, les nématodes et les microorganismes. Ce choix a été motivé rendre les différents axes du diagramme indépendants les uns des autres. Pour chacune des pages, le diagramme du dessous résume le diagnostic sur l'indicateur de synthèse correspondant en trois catégories : vert : bon état ; orange : état à surveiller ; rouge : état à améliorer.

A l'échelle de l'ensemble du réseau, tous les sols présentant une composante biologique. Entre 25% et 30% des parcelles échantillonnées présentaient un patrimoine biologique/une assurance écologique et/ou une fertilité biologique en bon état ; mais 15% à 20% des parcelles échantillonnées étaient à améliorer pour au moins un des deux indicateurs de synthèse. Le reste des parcelles était dans un état à surveiller (Figure 23). Cette observation indique qu'il est aujourd'hui important d'intégrer plus massivement les composantes biologiques dans l'évaluation de l'impact des pratiques agricoles et la conception des nouveaux systèmes de culture/production, ceci afin d'éviter la dégradation des parcelles présentant un état à

améliorer. Même si la taille du réseau ne permet pas de le tester statistiquement au regard du grand nombre de combinaisons de pratiques mises en œuvre, les résultats laissent supposer que l'amélioration de la composante biologique des sols pourrait se faire sans passer par des modifications abruptes des systèmes de culture.

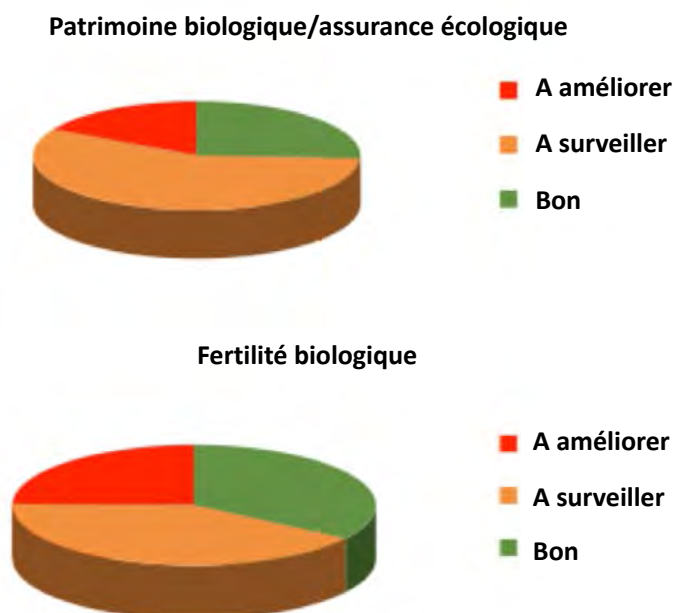


Figure 23. Distribution des parcelles entre les trois classes de diagnostic pour les indicateurs de synthèse patrimoine biologique/assurance écologique et fertilité biologique.

La démarche choisie (co-construction et restitutions) peut être jugée comme efficace au regard des effets à court terme observés. En effet, une enquête réalisée 18 mois après la fin du projet (E. Franc, Stage de Master 2, encadrement : L. Ranjard & N. Chemidlin Prévost-Bouré) a permis de mesurer l'impact du programme (n=66) : 97% des enquêtés intègrent la biologie des sols dans leurs questionnements et 60% ont engagé des changements de pratiques (gestion des couverts végétaux, réduction du travail du sol ou des intrants, rotations culturales). Aujourd'hui, la démonstration de l'opérationnalité du tableau de bord et des formations étant faite, les enjeux sont focalisés sur leur déploiement massif sur un réseau participatif élargit afin de couvrir au maximum les expérimentations menées à la ferme/au domaine par les acteurs du monde agricole.

Chapitre 4 – Perspectives de recherche

1. Projet scientifique de l'équipe BIOCOM

L'équipe BIOCOM "Distribution spatiale, dynamique et traduction fonctionnelle de la biodiversité des communautés microbiennes telluriques" créée en 2007 se compose actuellement de cinq chercheurs permanents (2 DR et trois MC), de deux personnels techniques auxquels s'associent généralement 2 à 3 personnels non permanents.

Le projet scientifique actuel de l'équipe BIOCOM s'inscrit dans un contexte où sociétés humaines dépendent plus que jamais des biens (patrimoine biologique, surfaces, aliments, énergie, matériaux tels que la terre ou le bois) et des services écosystémiques (cycles biogéochimique, régulation du climat, cycle de l'eau...) liés aux sols vu leur croissance démographique et leurs besoins énergétiques. Alors que les sols font face à des menaces de plus en plus nombreuses (érosion, baisse de fertilité, pertes en biodiversité...), il y a urgence à gérer durablement les ressources en sols. Cette gestion durable doit associer un fonctionnement du sol permettant de répondre aux besoins humains en maintenant/améliorant la qualité des sols. Les communautés microbiennes des sols, objets d'étude de l'équipe, sont reconnues comme centrales dans le fonctionnement des sols : leur biodiversité assure diversité, intensité et stabilité des fonctions biologiques. Par ailleurs, en plus de leur valeur patrimoniale, les communautés microbiennes des sols sont reconnues comme des bioindicateurs d'impact de l'usage des sols. Malgré tout, elles se doivent encore de devenir incontournables dans les approches choisies pour la gestion durable des ressources en sols. Pour cela, il faut affiner les connaissances sur les communautés microbiennes des sols et donner les moyens aux utilisateurs des sols de les prendre en compte dans leur gestion des sols.

Fort de ce constat, notre projet de recherche consiste en l'étude de la distribution spatiale et de la dynamique de la biodiversité des communautés microbiennes telluriques pour sa traduction en fonctionnement biologique du sol. Il est structuré en trois axes : 1) Étudier les processus et les filtres de la distribution spatiale des communautés microbiennes du sol à différentes échelles (de l'agrégat au territoire national) ; 2) Étudier le rôle de la diversité microbienne dans le fonctionnement biologique du sol ; 3) Développer et proposer des moyens opérationnels aux utilisateurs des sols pour évaluer la qualité microbiologique du sol et identifier comment orienter les communautés microbiennes indigènes pour optimiser les

Chapitre 4

services agro-écosystémiques fournis par le sol. La démarche associée repose sur le développement de concepts d'écologie des communautés microbiennes en s'appuyant sur la caractérisation sans à priori de leur diversité taxonomique et fonctionnelle par des outils de métagénomique environnementale sur l'ADN directement extrait du sol. Des partenariats publics ou privés, nationaux et internationaux permettent sa mise en œuvre à différentes échelles spatiales (de l'agrégat au territoire national) *via* des projets de recherche collaborative et participative. L'association de cette démarche à des approches de modélisation permet de produire des outils opérationnels adossés à des référentiels, outils qu'une offre de formation permet de transférer aux utilisateurs des sols.

Depuis le 1^{er} Mars 2018, j'anime le collectif de l'équipe BIOCUM. Sa double mission de recherche et de formation s'inscrit dans un contexte socio-économique qui évolue. Répondre aux enjeux agricoles en matière de gestion des sols reste essentiel mais cela doit s'intégrer au développement durable des filières et des territoires formés d'une mosaïque d'espaces agricoles et urbains. A court terme, ce changement d'échelles marquera une étape importante pour l'équipe qui déploiera ses axes de recherche sur : un nouveau milieu - les sols urbains ; une part croissante de réseaux de recherches participatives. L'étude des sols urbains s'appuiera sur des partenariats mais se devra d'être portée par un recrutement de chercheur au sein de l'équipe apportant ses compétences en écologie, en métagénomique et en statistiques. Le développement des recherches participatives permettra de massifier l'acquisition des données pour affiner les référentiels et de développer de nouveaux outils opérationnels qui pourront s'intégrer à des portails de services à l'agriculture et aux territoires (e.g. Portail DRIAS pour les services climatiques). Pour atteindre cet objectif, il est essentiel de renforcer les forces techniques de l'équipe (TFR) à court terme pour assurer l'acquisition des données en quantité et qualité ; et à moyen terme les compétences en modélisation de systèmes complexes en recherche et en formation pour assurer le développement des outils et des services. Ce renforcement des missions de recherche viendra supporter les missions de formation de l'équipe. Sur le plan académique, l'implication de l'équipe dans les formations ingénieur et universitaires et un renforcement de la formation doctorale (HDR, accueil de doctorants) permettront de participer à la formation pour et par la recherche, notamment d'ingénieurs-docteurs d'AgroSup Dijon. Sur le plan finalisé, l'équipe BIOCUM répond à une demande croissante de transfert

technologique et de formation aux nouveaux outils de diagnostic de qualité des sols par les acteurs du monde agricole. A court terme, cette réponse sera maintenue mais devra évoluer au regard des avancées techniques et de l'importance des demandes.

2. Projet de recherche au sein de l'équipe BIOCOM

Mon projet de recherche au sein de l'équipe BIOCOM s'inscrit dans la nécessité de mieux comprendre les déterminants de la diversité microbienne pour accompagner une gestion durable des sols. Plus précisément, mon objectif est de comprendre les dynamiques spatio-temporelles des communautés microbiennes des sols pour les modéliser et développer des outils opérationnels transférables auprès des utilisateurs des sols (axes 1 et 3 du projet de l'équipe BIOCOM). Ce projet se justifie au regard des avancées en écologie microbiennes. D'une part, les études d'écologie spatiale démontrent la structuration spatiale des communautés microbiennes du sol sous la dépendance de processus déterministes et neutres (Hanson *et al.*, 2012; Powell *et al.*, 2015) ; tout en soulignant le rôle des interactions biotiques dans cette structuration (Barberán *et al.*, 2012, [4]). Néanmoins, le caractère statique des études d'écologie spatiale ne permet pas d'évaluer si la distribution spatiale des communautés microbiennes et les processus associés varient dans le temps, réduisant les capacités de modélisation des communautés microbiennes. D'autre part, les études temporelles des communautés microbiennes des sols démontrent que leur dynamique temporelle est sous la dépendance de la diversité végétale (Buscardo *et al.*, 2018) et de la succession des pratiques agricoles (Tardy *et al.*, 2016 ; Ashworth *et al.*, 2017 ; Degruene *et al.*, 2017). Néanmoins, le caractère localisé de ces études limite la généralité de ces conclusions et leur déploiement à l'échelle des paysages agricoles ou du territoire national. Au final, ces limites restreignent leur transfert des connaissances acquises auprès des utilisateurs de sols.

Dans ce contexte, j'articulerai mon projet de recherche suivant un axe fondamental, un axe appliqué et un axe de transfert :

- 1) Axe fondamental : Comprendre la dynamique spatio-temporelle de la diversité microbienne des sols à l'échelle du paysage agricole;

- 2) Axe appliqué : Développer un Outil d'Aide à la Décision pour la gestion des communautés microbiennes des sols ;
- 3) Axe de transfert : Transférer aux utilisateurs des sols les connaissances en écologie microbienne des sols et les outils associés pour la gestion des sols.

2.1 Axe fondamental : Comprendre la dynamique spatio-temporelle de la diversité microbienne des sols à l'échelle du paysage agricole

Cet axe fondamental visera à : 1) Caractériser la dynamique spatio-temporelle des communautés microbiennes des sols ; 2) Évaluer la stabilité dans le temps des processus écologiques et des filtres environnementaux impliqués dans la structuration spatiale des communautés microbiennes des sols. Pour cela, j'associerai des approches de métagénomique environnementale pour la caractérisation des communautés microbiennes des sols au monitoring d'échantillonnages spatialisés. Cette stratégie est envisageable au regard des évolutions techniques en métagénomique environnementale et en bioinformatique qui permettent de traiter des échantillons à haut-débit. A court et moyen terme, je déploierai cette stratégie à l'échelle du paysage en m'appuyant sur deux dispositifs :

- le paysage agricole de Fénay qui couvre 1200ha et dont le monitoring a été initié en 2011, poursuivi en 2016 et sera réalisé une nouvelle fois en 2018 (4.2.).
- l'aire urbaine de Dijon Métropole qui couvre 3350 km² dont une majorité de terres agricoles. Elle fera l'objet d'un monitoring dans le cadre du projet "Dijon Métropole, territoire modèle du système alimentaire durable de 2030" dans le cadre de l'appel à projets "Territoires d'Innovation - Grande Ambition" (financement Caisse des Dépôts et Consignations, Chef de file : Dijon Métropole, Coordination Scientifique : P. Lemanceau, INRA). Plus précisément, ce monitoring sera réalisé au sein de l'action "Gestion des Sols" (Coordination L. Ranjard (INRA) & C. Claveirole (FNE, CESE)) qui vise à adapter l'utilisation des sols à leurs caractéristiques et à leurs contraintes ; et pour laquelle je serai responsable des sols "ruraux", *i.e.* hors de l'agglomération Dijon Métropole.

Chapitre 4

Ces dispositifs permettront d'identifier les échelles de temps auxquelles les communautés microbiennes du sol varient spatialement, de produire les premières cartographies dynamiques des communautés microbiennes des sols et d'évaluer la stabilité des processus écologiques et des filtres environnementaux (physico-chimie des sols, pratiques agricoles, successions végétales) impliqués dans la structuration spatiale des communautés microbiennes du sol. En plus des filtres environnementaux abiotiques ou anthropiques, le monitoring d'échantillonnages spatialisés permettra de gagner en connaissances sur les interactions biologiques dans les sols. La comparaison de réseaux d'interactions dans le temps sur un même dispositif permettra de mieux caractériser les interactions biologiques (synergies, antagonismes) au sein des communautés microbiennes des sols et d'évaluer leur stabilité vis-à-vis des pratiques agricoles. A moyen terme, ces interactions pourront être intégrées comme un filtre structurant les communautés microbiennes des sols. La généralité des résultats sera évaluée par confrontation à d'autres études effectuées à l'échelle paysagère (paysage agricole de Naizin, INRA Rennes) et de monitoring dans le cadre du Réseau d'Expérimentation et de Veille à l'innovation Agricole (REVA, organisme chef de file : Observatoire Français des Sols Vivants, collaborations AgroSup Dijon, ESA Angers, ISARA, IFV, CA49, CA71, CA 21, CA58, CA89) : Réseau participatif national d'agriculteurs de monitoring (tous les 2 à 5 ans) des parcelles à l'aide du tableau de bord de bioindicateurs développé dans le programme AgrInnov (5.2).

Cette étude renforcera mes collaborations avec plusieurs membres de mon équipe (L. Ranjard, S. Terrat, S. Dequiedt, B. Karimi) et de l'UMR Agroécologie (Plateforme GenoSol, pôle GESTAD), mais aussi avec l'Unité de Services InfoSol de l'INRA d'Orléans (D Arrouays, C Jolivet, N Saby) et le Génoscope (P Wincker, C Cruaud). Elle permettra de renforcer mes collaborations avec des partenaires de recherche locaux et nationaux (Université de Bourgogne Franche Comté ; Supagro Montpellier ; CNRS ; MNHN ; UMR SAS : V. Viaud ; ESA Angers : M. Cannavacciuolo ; ISARA Lyon : J.-F. Vian), des acteurs du monde agricole (Chambres d'Agriculture : J. Halska, CA71 ; V. Riou, CA 49 ; Coopératives) et du monde associatif (FNE ; Observatoire Français des Sols Vivants : E. D'Oiron). Par ailleurs, cette étude constituera un tremplin pour développer à court terme des collaborations internationales. En effet, ces travaux de monitoring font l'objet d'un intérêt particulier des chercheurs travaillant sur la gestion durable des sols (Université de Sydney :

Budiman Minasny, Professeur en sciences des sols et en modélisation des paysages ; Alexander McBratney, Directeur de l'Institut d'Agriculture de Sydney) et en écologie microbienne (Leho Tedersoo, Université de Tartu, Estonie).

2.2 Axe appliqué : Développer un Outil d'Aide à la Décision pour la gestion des communautés microbiennes des sols

Cet axe de recherche appliquée vise à modéliser la dynamique temporelle des communautés microbiennes des sols pour construire un outil d'aide à la décision à destination des gestionnaires des sols, ceci sur la base des informations acquises dans le cadre de mon axe de recherche fondamentale. Vu l'important niveau de diversité des microorganismes du sol, leur forte variabilité spatiale et temporelle, et le grand nombre d'interactions entre groupes microbiens, des modèles spatialement explicites et des simulations seront mis en œuvre pour modéliser la dynamique des communautés microbiennes des sols face aux changements de pratiques agricoles et en appréhender le devenir (Kant, 2015, Groffman & Jones, 2000). L'élaboration de ces modèles est aujourd'hui possible au travers des approches APSF (Marilleau et al., 2008) et des algorithmes développés dans Coupling4MAS (Hassoumi, 2015) permettant de combiner des modèles très différents dans leur concept, leur formalisme et leur fonctionnement.

Ces modèles aboutiront à une sortie finalisée puisqu'ils constitueront la base d'un Outil d'Aide à la Décision (OAD) pour la gestion des communautés microbiennes des sols. Cet OAD viendra compléter le tableau de bord développé dans le cadre du programme AgrInnov en répondant à une attente forte des acteurs du monde agricole d'évaluer à priori les modifications de pratiques qu'ils envisagent. Il sera construit et validé au travers d'une approche de modélisation participative (AGILE et Commod; Etienne, 2010) associant différents acteurs (scientifiques et du monde agricole) au sein de groupes pluridisciplinaires. Le choix de cette approche est motivé par la nécessité d'adapter l'OAD aux besoins et aux enjeux des acteurs et des futurs utilisateurs, et de favoriser son acceptation (Becu et al., 2015a ; 2015b ; 2016). Une fois construit, cet OAD sera évalué pour sa généricité et son opérationnalité à l'échelle de l'aire urbaine de Dijon métropole dans le cadre du projet "Dijon Métropole, territoire modèle du système alimentaire durable de 2030" ;

et à l'échelle nationale dans le cadre du Réseau d'Expérimentation et de Veille à l'innovation Agricole (REVA).

Cette étude renforcera ma collaboration avec L. Ranjard et me permettra de développer une collaboration avec N. Marilleau (UMI UMMISCO, IRD Bondy, et UMR FEMTO-ST, Université Bourgogne-Franche-Comté) dans le cadre d'un projet doctoral. Elle renforcera les collaborations initiées lors de la première campagne, en particulier avec l'INRA d'Orléans (D. Arrouays, C Jolivet & N. Saby), et s'appuiera sur des interactions fortes au sein de l'UMR Agroécologie (Plateforme Génosol, pôle GESTAD) et sur les collaborations développées au sein du REVA notamment avec l'ESA Angers (M. Cannavacciulo), l'ISARA de Lyon (J.F. Vian), l'Observatoire Français des Sols Vivants (E. D'Oiron) ou les chambres d'agriculture (J. Halska, CA71 ; V. Riou, CA 49).

3. Axe de transfert : Transférer les connaissances en écologie microbienne des sols et les outils associés pour la gestion des sols

Faisant écho aux deux premiers axes de mon projet de recherche, mon axe de transfert se positionne à la fois au niveau académique et au niveau opérationnel.

3.1 Transfert académique

Le transfert académique s'inscrit dans mes activités de formation au sein d'AgroSup Dijon. A travers la constitution d'un collectif d'enseignants-chercheurs de plusieurs départements d'enseignement d'AgroSup Dijon (Agronomie, Agroéquipements, Elevage, Environnement ; Sciences Humaines et Sociales ; Sciences de l'Ingénieur et des Procédés), j'ai proposé une dominante de 3^e année ingénieur pour la gestion des ressources naturelles (sol, eau, biodiversité). Cette dominante intitulée "Ressources, Données, Diagnostic, Changements Climatiques" (R²D²C) ouvrira en septembre 2018. Elle formera des ingénieurs opérationnels capables de proposer des stratégies de gestion des ressources naturelles (sol, eau, biodiversité) dans un contexte de changements climatiques en s'appuyant sur des outils d'évaluation et de diagnostic, notamment les bioindicateurs microbiens que j'ai contribué à développer au fil de mes activités de recherche.

3.2 Transfert opérationnel

Le transfert opérationnel est orienté sur l'utilisation des bioindicateurs microbiens pour la gestion des sols au travers du tableau de bord AgrInnov. Il se décline en trois actions reliées chacune à un niveau d'organisation : les filières de production, les acteurs, et les territoires.

A l'échelle de la **filière**, je coordonne le projet IFEP (2016-2020, financements CIGC et I-SITE Bourgogne Franche-Comté, partenariat CIGC, UMR Chrono-environnement, IDELE, Conservatoire Botanique National de Franche-Comté) qui vise à évaluer, en filière Comté, les impacts de la fertilisation des prairies sur leur biodiversité et les transferts de bactéries et de contaminants du sol au lait. S'appuyant sur un réseau de recherche collaborative de 45 fermes le long de l'arc jurassien, il permettra d'aboutir à des recommandations de fertilisation des prairies préservant l'environnement de production et maintenant le lien entre fromage et terroir. Il pourra servir d'exemple à d'autres AOP pour allier développement de la filière et préservation de l'environnement de production.

Au niveau des **acteurs**, je mettrai en œuvre deux actions. D'une part, je m'implique dans le transfert technologique des outils de diagnostic auprès des laboratoires d'analyse. Pour cela, je participe au projet Agro-Eco Sol (Chefs de File : Aurea Agrosiences, INRA et Arvalis Institut du Végétal ; Coordination scientifique : L. Ranjard) au titre de mon expertise sur les bioindicateurs microbiens. Ce projet est financé dans le cadre des Projets d'Investissement d'Avenir 3 de l'Etat qui vise à industrialiser les méthodes d'analyse des bioindicateurs pour pouvoir les proposer en prestation; constituer une base de données pour affiner les diagnostics ; construire un conseil basé sur ces bioindicateurs ; et former les utilisateurs. Ceci facilitera l'utilisation des bioindicateurs microbiens, notamment en réduisant les coûts analytiques. En parallèle, je poursuivrai mes actions opérationnelles de déploiement des indicateurs microbiens pour le diagnostic de la qualité microbiologique des sols en agriculture. Ces actions seront réalisées dans le cadre du REVA par la formation professionnelle et le transfert d'outils. Ce réseau de recherche participative pour le monitoring de la qualité biologique des sols agricoles (tous les 2 à 5 ans) permettra d'identifier les pratiques agricoles améliorant la qualité biologique des sols dans une grande diversité de contextes pédoclimatiques. Il permettra aussi de disséminer l'OAD auprès des agriculteurs et des viticulteurs.

Chapitre 4

A l'échelle du **territoire**, je suis impliqué dans le projet "Dijon Métropole, territoire modèle du système alimentaire durable de 2030 au sein de l'action "Gestion des sols". Dans ce cadre, mon rôle sera d'assurer la coordination entre les différentes tâches sur les sols "ruraux" qui couvrent des thématiques allant de la caractérisation des sols et de la biodiversité au stockage de carbone, à la qualité des eaux et à l'économie ou la politique foncière. S'appuyant sur une démarche de recherche participative, cette action sera aussi un outil sans pareil pour disséminer l'OAD auprès des utilisateurs des sols. Pour cela, je collaborerai étroitement avec mes collègues L. Ranjard et P.-A. Maron et avec C. Claveirole (FNE, CESE), mais aussi avec des partenaires de la recherche locaux ou nationaux (Université de Bourgogne Franche Comté, Supagro Montpellier, CNRS, MNHN), des acteurs du monde agricole (Chambres d'Agriculture, Coopératives) et du monde associatif (Observatoire Français des Sols Vivants).

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ATLAS FRANÇAIS DES BACTÉRIES DU SOL



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RESEARCH ARTICLE

Mapping and predictive variations of soil bacterial richness across France

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Abstract

Although numerous studies have demonstrated the key role of bacterial diversity in soil functions and ecosystem services, little is known about the variations and determinants of such diversity on a nationwide scale. The overall objectives of this study were i) to describe the bacterial taxonomic richness variations across France, ii) to identify the ecological processes (*i.e.* selection by the environment and dispersal limitation) influencing this distribution, and iii) to develop a statistical predictive model of soil bacterial richness. We used the French Soil Quality Monitoring Network (RMQS), which covers all of France with 2,173 sites. The soil bacterial richness (*i.e.* OTU number) was determined by pyrosequencing 16S rRNA genes and related to the soil characteristics, climatic conditions, geomorphology, land use and space. Mapping of bacterial richness revealed a heterogeneous spatial distribution, structured into patches of about 111km, where the main drivers were the soil physico-chemical properties (18% of explained variance), the spatial descriptors (5.25%, 1.89% and 1.02% for the fine, medium and coarse scales, respectively), and the land use (1.4%). Based on these drivers, a predictive model was developed, which allows a good prediction of the bacterial richness (R^2_{adj} of 0.56) and provides a reference value for a given pedoclimatic condition.

Introduction

Numerous studies performed over the last two decades in the field of microbial ecology have focused on variations of the soil microbial diversity under different environmental conditions to better understand its regulation and predict the impact of perturbations [1±4]. These works were justified by the lack of knowledge about the determinants of microbial diversity in space and time, but also by the growing awareness of the key role of soil microbial diversity in soil functions (C and N recycling, pathogen management, bioremediation. . .) [1,5±8] and the supply of ecosystem services. In this context, we have therefore accumulated a huge number of

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studies dealing with precise perturbations on a plot scale (e.g. [1]). Soil microbial community as a whole and how it varies has already been examined over regional (e.g. [9]), territorial (e.g. [10]) or continental scales (e.g. [4,11]) by several studies. One of the main pioneer works was performed by Fierer & Jackson (2006) who described soil bacterial diversity on a continental scale by applying DNA fingerprinting to 98 soils sampled along an environmental transect from the north to the south of America [12]. They demonstrated that bacterial diversity was closely related to soil characteristics and especially the pH, as recently confirmed in other studies [10,13]. But, more recently, other studies [9,11,14] have demonstrated the prevalence of other parameters (like climate, geomorphology or land use) on bacterial distributions across regional or global scales. For example, Maestre et al., (2015) highlighted that aridity indirectly impacted the diversity and abundance of soil *bacteria* and *fungi* by strongly affecting soil pH, soil organic C content, and total plant cover using data from 80 dryland sites across the globe [11]. Another study, based on a European soil transect (72 sites showed that soil pH was the main driver of soil bacterial community structure), and established a predictive model of soil bacterial community structure allowing to draw a map at the European scale based solely on this soil parameter [15]. In the same way, two recent studies have developed statistical models to build global spatially explicit predictions of soil microbial biomass [16,17].

However, most of the studies that compared soil microbial diversity and composition were conducted in very different types of ecosystems and soils (generally chosen with *a priori*), which could have facilitated community discrimination and exacerbated the relationship with contrasting environmental filters (soil characteristics, climatic conditions, land cover etc.). In addition, reducing the number of environmental parameters examined and/or their range of variation can lead to contradictory results concerning, for example, the influence of climatic conditions on soil bacterial diversity [12,18]. Drawing a robust conclusion, as to the ecological processes involved (deterministic *vs* neutral processes) or the hierarchy of environmental filters driving soil microbial diversity on a nationwide scale, currently seems impossible from these studies. However, more recently some studies using large soil sampling on a regional scale started to decipher the ecological processes (deterministic *vs* neutral processes) driving soil microbial diversity. Recently, a study showed that habitat turnover was the primary driver of bacterial community turnover, but its importance decreased with increasing isolation [19]. These studies paved the way for the importance of conducting new extensive studies on a nationwide scale with high resolution sampling and without *a priori* to improve the robustness and general applicability of the conclusions and then the understanding of soil microbial community regulation.

In France, the French Soil Quality Monitoring Network (Réseau de Mesures de la Qualité des Sols = RMQS) represents the most extensive and without *a priori* soil sampling survey available to date and fulfils most of the above-cited requirements [20]. It consists of a systematic sampling grid (16 x 16 km) extending over the whole of France with 2,173 sites covering an area of $\approx 5.3 \times 10^5$ km² with a huge diversity of soil physico-chemical characteristics, plant cover, land use, geomorphology and climatic conditions and coupled with an extensive collection of corresponding environmental data (Fig 1) [21]. In previous studies, by applying molecular tools to characterize the microbial communities in all RMQS samples, we demonstrated that soil molecular microbial biomass was heterogeneously distributed on the scale of France with biogeographical patterns of about 160 km radius, mostly driven by the soil texture, the pH, the organic carbon content of the soil and by the land use with a negative impact of agricultural land use conversely to natural or semi natural land use [22,23]. Based on these drivers we developed an original predictive polynomial model that provides a reference value for microbial biomass for a given pedoclimatic condition, which can then be compared with the corresponding measured value to provide a robust diagnosis of soil microbiological status

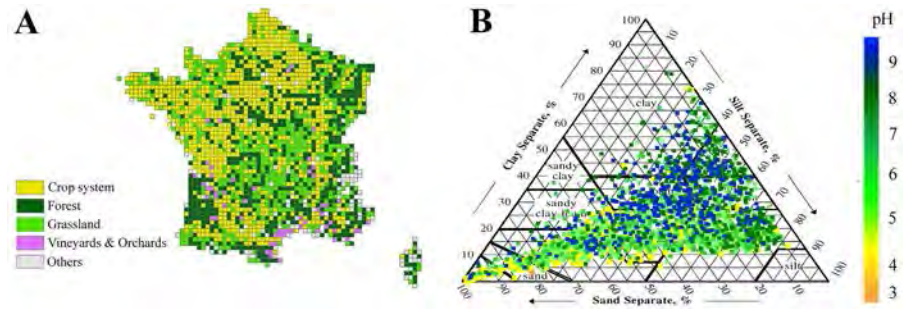


Fig 1. Locations, land uses and texture of sampling sites from the French Soil Quality Monitoring Network (RMQS). (A) Location of sampling sites in the systematic sampling grid of the French Soil Quality Monitoring Network (RMQS) criss-crossing the whole French territory. Colour legend indicates the various types of land use encountered in France on this scale. "Others" land use corresponds to sites impossible to sample which corresponded to inaccessible sites (mountain, sea, etc.) or sites without natural soils (urban zone, rocky zone...). (B) Distribution of the RMQS soils in the USDA soil texture triangle. Colour legend from yellow to blue represents the soil pH of each RMQS soil.

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[17]. By applying a DNA-fingerprinting approach, we also proved a heterogeneous distribution of soil bacterial community structure, which was independent of soil microbial biomass distribution but driven by soil physico-chemical properties and land use [22]. By comparing estimates of the taxa-area relationship with habitat heterogeneity, we demonstrated that the turnover rate of bacterial diversity in soils on a nationwide scale was (i) highly significant and strongly correlated with the turnover rate of soil habitat [24], and (ii) driven by dispersal limitation as well as environmental selection, this latter including soil and land use properties [13]. Since all these studies were based on quantitative and community structure characterization of bacterial communities, they did not provide information about bacterial diversity in terms of richness, evenness and taxonomic composition.

The aim of the present study was to use the RMQS monitoring network to evaluate the variations and to decipher the spatial patterns of bacterial richness in soils across French national territory. More precisely, our study focused on bacterial taxonomic richness (in terms of number of Operational Taxonomic Units or OTUs at 95% of sequence similarity, corresponding roughly to the *genus* level) [1,6]. Bacterial richness was determined in all 2,173 soils samples of the RMQS by using a pyrosequencing of bacterial 16S rRNA genes directly amplified from soil DNA. Geostatistics was applied to these data to provide the first comprehensive map of soil bacterial richness variation along the environmental gradients encountered in France. The ecological processes structuring the variation of bacterial richness were identified and ranked by variance partitioning analysis. Finally, a statistical predictive model was developed according to the environmental filters identified. This model represents an operational tool highly complementary with the predictive model of soil molecular microbial biomass developed previously [16,17] to establish a comprehensive diagnosis of the soil microbiological status (in terms of abundance and diversity of soil microorganisms).

Materials and methods

Soil sampling strategy

Soil samples were obtained from the French Soil Quality Monitoring Network ("Réseau de Mesures de la Qualité des Sols" = RMQS) which is a soil monitoring network based on a 16 km regular grid across the 550,000 km² French territory [25]. The RMQS includes 2,173

Table 1. Statistical description of environmental parameters for RMQS soil samples. These values are based on the 1,798 sites analyzed. CEC: cation-exchange capacity; ETP: evapotranspiration.

Soil properties (unit)	Minimum	First Quartile	Median	Mean	Third Quartile	Maximum
pH water	3.70	5.40	6.23	6.42	7.80	8.90
Organic Carbon (g.kg ⁻¹)	2.57	13.60	19.80	26.08	30.70	243.00
Total Nitrogen (g.kg ⁻¹)	0.11	1.180	1.75	2.20	2.71	16.00
C:N	6.26	9.67	10.56	12.10	13.27	52.72
Total Calcium Carbonate (g.kg ⁻¹)	0.50	0.50	0.50	56.19	12.38	866.00
Available phosphorous (g.kg ⁻¹)	0.001	0.014	0.036	0.053	0.077	1.110
CEC (Cmol + kg ⁻¹)	0.25	5.84	10.30	14.32	20.30	70.10
Clay (g.kg ⁻¹)	5.0	154.0	213.0	248.9	325.8	819.0
Silt (g.kg ⁻¹)	2.0	280.0	406.5	410.6	540.8	819.0
Sand (g.kg ⁻¹)	7.0	152.0	287.0	340.4	502.0	985.0
Total Cd (g.100g ⁻¹)	0.01	0.12	0.20	0.30	0.35	4.10
Total Cu (g.100g ⁻¹)	0.50	8.77	13.90	19.93	22.10	491.00
Total Ni (g.100g ⁻¹)	0.50	11.72	19.70	25.20	31.50	1,530.00
Total Pb (g.100g ⁻¹)	3.06	21.40	28.20	32.97	38.00	624.00
Total Zn (g.100g ⁻¹)	2.50	43.83	64.32	74.55	90.20	1,080.00
Total K (g.100g ⁻¹)	0.02	1.06	1.44	1.60	2.01	5.40
Elevation (m)	-3.0	106.0	194.5	331.1	388.8	2,540.0
Mean Annual ETP (mm)	43.38	50.45	54.31	55.67	58.61	96.11
Mean Annual Rain (mm)	45.78	62.66	71.89	76.77	84.11	183.71
Mean Annual Temperature (°C)	-2.32	9.93	10.72	10.66	11.73	15.49

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monitoring sites, collected between 2000 and 2009, each located at the centre of a 16 x 16 km cell (Fig 1). All sites have been geo-positioned with a precision <0.5m and the soil profile, site environment, climatic factors, vegetation and land use described (see Table 1). In the middle of each 16 x 16 km square, 25 individual core samples were taken from the topsoil (0±30 cm) using an unaligned sampling design within an area of 20 x 20 m. The core samples were bulked to obtain a composite sample for each RMQS site. The soil samples were gently air-dried, sieved to 2mm and then stored at -40°C before analysis [23]. Physico-chemical parameters were measured for each composite soil, e.g. particle-size distribution, pH water, organic C, N, C/N ratio, soluble P contents, calcareous, cation exchange capacity (CEC) or exchangeable cations (Ca, Mg). Physical and chemical analyses are available for 2,131 soils and were performed by the Soil Analysis Laboratory of INRA (Arras, France, <http://www.lille.inra.fr/las>). Available climatic data for the RMQS were annual rain, evapotranspiration and temperature. These data were obtained for each node of a 12 x 12 km grid defined by Meteo-France, obtained by interpolating observational data using the SAFRAN model [26]. Obtained measures for the period 1992±2004 were then averaged, to integrate all transitory effects into one value corresponding to a global effect of climate on soil microbial communities. Finally, the RMQS site-specific data were linked to the climatic data by finding for each RMQS site on the grid of 16 x 16 km the closest node within the 12 x 12 km climatic grid. Land use was recorded according to the coarse level of the CORINE Land Cover classification (<http://land.copernicus.eu/pan-european/corine-land-cover>), which consists of a rough descriptive classification into five classes: forests, croplands, grasslands, others and perennial crops (corresponding to vineyards and orchards). All these data were available in the DONE-SOL database [21].

Molecular characterization of bacterial community diversity

Soil DNA extraction and purification. Microbial DNA was extracted and purified from 1g of the 2,173 composite soils (composed of a bulk of 25 individual core soils) sampled in each RMQS site, using the G_nS-GII procedure as described previously [27]. Crude DNA extracts were quantified by agarose gel electrophoresis stained with ethidium bromide and using calf thymus DNA as standard curve [23]. Crude DNA was then purified using a MinElute gel extraction kit (Qiagen, France) and quantified using a QuantiFluor staining kit (Promega, USA), prior to further investigations.

PCR amplification and pyrosequencing of 16S rRNA gene sequences. A 16S rRNA gene fragment targeting the V3-V4 regions to characterize bacterial diversity was amplified using the primers F479 (5' -CAGCMGCYGCNGTAANAC-3') and R888 (5' -CCGYCAATTCMTTTRAGT-3') [27]. 2,132 soil samples were successfully amplified from the 2,173 DNA soil samples. The 16S PCR products were then purified using a MinElute PCR purification kit (Qiagen, Courtaboeuf, France) and quantified using the QuantiFluor staining kit (Promega, USA). A second PCR of 7 cycles was then duplicated for each sample under similar PCR conditions, with purified PCR products as matrix (7.5ng of DNA were used for a 25µl mix of PCR) and dedicated fusion primers ('F479/AdaptorB', 'R888/MID/AdaptorA') integrating needed adaptors, keys and multiplex identifiers at 5' extremities. All duplicated PCR products were then pooled, purified using a MinElute PCR purification kit (Qiagen, Courtaboeuf, France), and quantified using the QuantiFluor staining kit (Promega, USA). For all libraries, equal amounts from 30 samples were pooled, and then cleaned to remove excess nucleotides, salts and enzymes using the Agencourt AMPure XP system (Beckman Coulter Genomics). 100µl of TE buffer (Roche) was used for the elution. Pyrosequencing was then carried out on a GS FLX Titanium (Roche 454 Sequencing System) by Genoscope (Evry, France).

Bioinformatics sequence analysis. Bioinformatic analyses were done using the G_nS-PIPE developed by the GenoSol platform (INRA, Dijon, France) [28]. Chosen parameters for each step can be found in [S1 Table](#) and the details of all steps have been already described previously [27]. Regarding the filtering step, it was then carried out to check all single-singletons (reads detected only once and not clustered) were checked in order to eliminate PCR chimeras and large sequencing errors produced by the PCR and the pyrosequencing, based on the quality of their taxonomic assignments. More precisely, each single-singleton was compared with a dedicated reference database from the Silva curated database using similarity approaches (USEARCH), with sequences longer than 500 nucleotides, and kept only if their identity was higher than the defined threshold ([S1 Table](#)). Finally, the number of high-quality reads for each sample was normalized (*i.e.* 10,000 high-quality reads for each sample) by random selection to allow efficient comparison of the data sets and avoid biased community comparisons (see [S1 File](#)). A total of 1,798 soil samples were finally kept for subsequent analyses.

A post-processing filtering was then applied to this global dataset to account for potentially artefactual data. First all the homogenized high-quality reads from all samples (encompassing a total of 17,980,000 reads) were merged and aligned. Then, as the analysis of microbial community richness relies on the construction of similarity clusters (called OTUs), we chose here to use OTUs to examine the distribution of 16S rRNA gene sequences in our datasets. However, there is no single best definition of 'species', 'genus' when this approach is used, because of controversy about thresholds of similarity allowing clear differentiation of taxonomic units [29]. Moreover, a recent study regarding the diversity of bacterial genomes demonstrated that when the standard threshold of 97% is used, some species can fall to different OTUs due to intragenomic or intraspecific differences [30]. So, we decided to apply the 95% threshold of sequence similarity, usually considered as the 'genus' level. This clustering was realized with a

PERL program that groups rare reads to abundant ones, and does not count differences in homopolymer lengths. A post-processing step was then applied to remove all singleton OTUs that occurred only once in the overall dataset, and comprised only a singleton (reads detected only once after the dereplication step and not clustered) [27]. This post-processing step reduced the number of total OTUs from 205,590 to 92,571 (loss of 50%), but the number of reads only from 17,980,000 to 17,866,981 (loss of less than 1%). For each sample, the number of deleted reads with this step was 62 ± 60 on average (minimum: 10, maximum: 1,093). Finally, contingency tables of OTUs were obtained with the samples in lines and OTUs in columns, indicating the number of reads in each OTU for all samples. The retained high-quality reads were then used to determine OTU richness and rarefaction curves (see S2 File) [31]. All raw data sets are publicly available in the EBI database system (in the Short Read Archive) under project accession PRJEB21351.

Metadata analysis

Mapping using geostatistic. The geostatistical method of kriging was used to map microbial richness and to characterize their spatial variations [32]. More precisely, as the studied variable followed a normal distribution (Kolmogorov-Smirnov test, p -value = 0.2703 for Richness), no transformation was considered prior to modelling the spatial correlations (S1 Fig). In conventional geostatistical analysis, an estimate of a variogram model is computed based on the observations, which describe the spatial variation of the property of interest. This model is then used to predict the property at unsampled locations using kriging [32]. A common method for variogram estimation is first to calculate the empirical (so called experimental) variogram by the method of moments [33], and then to fit a model to the empirical variogram by (weighted) nonlinear least squares. We tried to fit several models and retained the one that minimized the objective function [34]. The validity of the best fitted geostatistical model was then assessed in terms of the standardized squared prediction errors (SSPE) using the results of a leave one out cross validation. If the fitted model was a valid representation of the spatial variation of the microbial property, then these errors would have a χ^2 distribution with a mean of 1 and median of 0.455 [35]. The mean and median values of the SSPE were also calculated for 1,000 simulations of the fitted model to determine the 95% confidence limits. The 'gstat' package of R software (version 3.2.2) was used for geostatistical analysis and kriging [36].

Variance partitioning. The relative contributions of soil physicochemical parameters, land use (forests: 492 sites, croplands: 740 sites, grasslands: 464 sites, perennial crops, corresponding to vineyards and orchards: 36 sites, and others: 36 sites), climatic conditions, geomorphology and space in shaping the patterns of soil bacterial richness and evenness were estimated by variance partitioning. The Principal Coordinates of a Neighbour Matrix approach (PCNM) was used to describe and identify the scales of spatial relationship between samples [37]. This PCNM method was applied to the geographic coordinates and only PCNMs with a significant Moran's index were selected for the variance partitioning analysis ($P < 0.001$). The spatial neighbourhood described by each PCNM was determined by the range of a Gaussian variogram models [38]. All quantitative (response and explanatory) data were standardized (centered and scaled) in order to have an approximated Gaussian and homoskedastic residual distribution. A two steps procedure was used to determine the environmental parameters significantly shaping bacterial richness and to limit over fitting and to exclude co-linear variables [39]. The first step consisted of a coarse selection of explanatory variables included in models minimizing the Bayesian Information Criterion (BIC) and maximizing the adjusted R^2 using the *regsubset* function ('leaps' package) [40]. In the second step, a forward

selection procedure was applied to the subset of explanatory variables to identify the model maximizing the adjusted R^2 [39]. Spatial descriptors were then selected from the model residuals [41] using the forward selection step only since all PCNM are linearly independent. The respective amounts of variance (*i.e.*, marginal and shared) for bacterial richness, were determined by canonical variation partitioning and the adjusted R^2 with Redundancy Analysis [39]. The statistical significance of the marginal effects was assessed from 1,000 permutations of the reduced model. All these analyses were performed with R (<http://www.r-project.org/>) using the *vegan* package.

Predictive modelling strategy. Three steps were assessed in order to find the best explanatory and most parsimonious model that explained the bacterial richness (response variable) as a function of soil physico-chemical characteristics and geographical coordinates (climatic data were not retained since they are rarely available, expensive to obtain, and limit the use of the model in a diagnostic approach [17]) (explanatory variables), i) selection of the significant explanatory variables, ii) selection of the best model form based on its predictive capacity and cross validation, and iii) sensitivity analyses of the model. For the first step, two tools were used to assess collinearity between the explanatory variables, namely correlation coefficients and variance inflation factors (VIF). Only those with a correlation coefficient ranging from -0.7 to 0.7 and with a $VIF \leq 4$ were considered in the modeling steps. The VIF values were calculated using the *vif* function in the *car* R package [42]. This selection step allowed the exclusion of highly collinear variables and defined a reduced explanatory dataset more comprehensive and of easier use for the following steps. Since the number of explanatory variables was large (less than 50), the best explanatory variables were selected by applying the exhaustive search method described by Miller, 2002 [43]. This approach involved using the *regsubsets* function in the *leaps* package in R [40]. The selection criteria were the Bayesian Information Criterion (BIC) and the adjusted coefficient of determination (R^2_{adj}) by minimizing the first and maximizing the second.

For the second step, the bacterial richness dataset was randomly divided into a modeling dataset (90% of the data, 1,618 soil samples) and a cross-validation dataset (about 10% of the data, 180 soil samples), selected by applying the KennardStone algorithm. The *kenStone* function of the *prospectr* package was used to determine the distribution of the modeling and cross-validation datasets. Different polynomial linear models were then compared, with different numbers and types of explanatory variables as well as different degrees. Model selection was therefore based on maximizing R^2_{adj} , while minimizing BIC and by cross-validating the model on the cross-validation dataset.

Since the basis of the model was linear regression, standardized regression coefficients (SRC) were used as sensitivity index, as classically reported in the literature [44]. The regression coefficients denoted by $\hat{\beta}$ were determined by ordinary least-squares regressions and provided information about the sensitivity of the model response to the various input-factors, and their combinations. SRC is equal to $(\sigma_{X_i}/\sigma_Y)*\hat{\beta}$, where σ_{X_i} and σ_Y are the standard deviation of inputs and output variables, respectively. The SRC values were determined using the *sensitivity* package in R [45]. With this approach, the sensitivity of the model to a given variable is high when the absolute value of SRC is high.

Results and discussion

This study provides an extensive compilation of bacterial richness from the soil environment i) to draw the first map of bacterial richness across France with over 1,700 geo-located samples, ii) to decipher the ecological processes (selection vs dispersal limitations) involved in such distribution and also iii) to elaborate an operational predictive model of bacterial richness

according to soil parameters. By applying pyrosequencing technology to soil DNA from all the composite soil samples in the French monitoring network, we were able to generate more than 17,980,000 16S rRNA sequences and to describe 92,571 different OTUs.

Soil bacterial richness variation and distribution across French national territory

Bacterial richness recovered from the 1,798 RMQS soils, ranged from 555 to 2,007 detected OTUs with an average value of 1,288 (± 207) OTUs and half of the RMQS soils harboured between 1,170 and 1,424 OTUs (S1 Fig). These results are in the same order of magnitude as those classically obtained in different soil environments, using comparable sequencing technology and sequencing depth [46,47]. Such a great variation might result from our extensive sampling strategy, which enabled various types of soil and land uses to be compared. Another consequence of this huge variability of soils and environmental parameters was that the cumulative number of different OTUs detected did not reach the saturation even when all the 1,798 RMQS soils studied were considered (S2 Fig).

In this study we provide the first national map of soil bacterial richness with its experimental and fitted variogram (Fig 2). The results of the 10-fold cross-validation gave a mean value of the SPPEs that is 1.021 and very close to the expected value, and a median value of 0.3922, both values falling within the 95% confidence interval. As indicated by the parameters of the Matérn function of the variogram, the observed (nugget / (nugget + sill)) ratio was high (= 0.73), suggesting that a large proportion of the variance was unexplained. Despite the rigorous standardisation of our molecular tools from soil DNA extraction to sequencing technology [27], the unexplained variance might be partly due to methodological variability. It might also be due to the large scale of the sampling scheme, which is unsuitable for detecting rough spatial process at small distance as previously suggested [48].

The map obtained revealed a heterogeneous distribution of bacterial richness, which was to a large extent spatially structured in geographical patterns defining more or less wider regions with hot- or cold-spots (Fig 2). The fitted model gave an effective range of 111.6 km revealing a large autocorrelation distance but smaller than those observed for molecular microbial

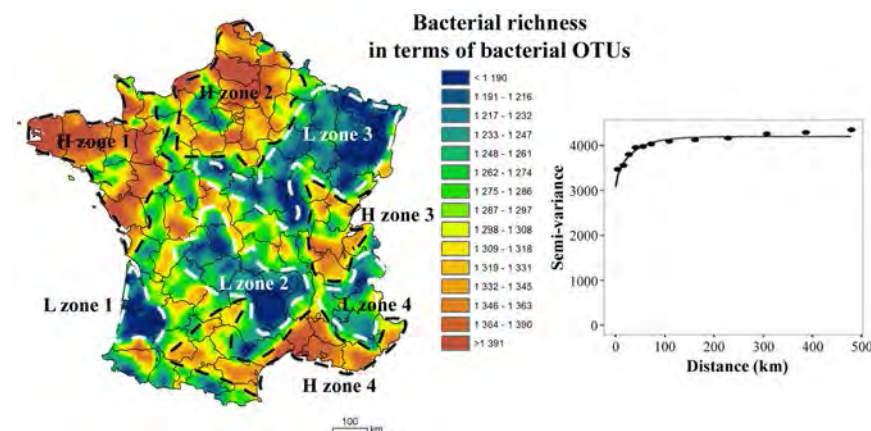


Fig 2. Mapping and robust variograms of soil bacterial richness on the scale of France. The colors indicate the extrapolated values expressed as OTU per soil sample. The L and H zones visually observed on the map correspond to Low and High bacterial richness zones on a regional scale, respectively. In the graph, points represent the experimental variogram, and continuous lines the Matérn models fitted by maximum likelihood method.

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biomass (160km) [23]. This difference confirmed that the abundance and diversity of soil microorganisms are not driven by the same filters as demonstrated at another spatial scale [49].

The scale of spatial variations of bacterial richness did not correspond to the French climatic distribution (Soil Atlas of Europe, climate p. 122) or to the presence of large natural barriers (mountain, sea. . .; Soil Atlas of Europe, elevation p. 121) [50]. On the other hand, the observed geographical patterns of bacterial diversity could be matched with large pedological patterns. The distribution of French soil types in terms of physico-chemical characteristics (<http://gissol.orleans.inra.fr/programme/bdgsf/carte.php>) corresponded to certain richness hot- or cold-spots, suggesting that these soil physico-chemical characteristics had a strong influence. For example, the cold-spots of OTUs located in North-East and in South-West (L-zone 1, and a part of L-zone 3, respectively, Fig 2) correspond closely to the most acidic soils in France (<http://www.gissol.fr/donnees/cartes>). In addition, distribution of bacterial richness patterns could correspond to the coarse level of land cover distribution described for France (Fig 1; <http://www.statistiques.developpement-durable.gouv.fr/clc/fichiers/>; Soil Atlas of Europe, land cover p. 123) [50]. The low number of bacterial OTUs recorded in Landes, Centre and North-East (L-zones 1, 2 and 3, respectively, Fig 2) could be related to the distribution of particular land covers, notably forest and grasslands, in these regions (Fig 1). In contrast, hot spots of bacterial richness seemed mainly to correspond to regions under crop systems, such as the Brittany, the North and the South around the Mediterranean (H-zones 1, 2 and 4, respectively). These observations imply that the autocorrelation distance might be partly driven by the influence of large patterns of soil types and coarse level of land cover distribution on bacterial diversity.

Ecological processes driving soil bacterial richness

Total variance was partitioned between five types of explanatory sets of environmental parameters: soil properties, land management, climate, spatial descriptors and interactions. Soil parameters, land management, climate and their interactions are linked with ecological processes derived from the deterministic theory and based on selection by the environment [51] whereas spatial descriptors can be partly related to variations in unmeasured environmental parameters [52] and/or linked with neutral processes such as dispersal limitation [53]. The variance partitioning approach revealed that the total amount of explained variance of bacterial richness was 48.2%, which is significantly lower than those observed at the landscape scale [49]. This difference might be due to the smaller variation in soil characteristics, climate and geomorphology on a landscape scale than on the scale of France. Variance partitioning indicated a significant influence ($P < 0.01$) of soil characteristics (18% of explained variance), spatial descriptors (8.2%), land use (1.4%), climatic conditions (0.4%), but not of geomorphology (Fig 3A). Interactions between soil characteristics, land use and climate represented also a large proportion of the explained variance (20.4%). These observations are congruent with recent studies evidencing the major effect of soil characteristics on bacterial richness, and consequently the high impact of selection processes (due to the influence of specific environmental parameters) shaping bacterial richness [4,46]. On the other hand, the influence of space might be partly related to variations in unmeasured environmental characteristics [52] but also suggests that dispersal limitation may be non negligible in shaping bacterial richness [13,54].

For each filter, within the sets of environmental and spatial descriptors, the marginal effect accounted for relatively small, but significant, proportions of the total variance (from 0.4% to 11%) due to the large number of parameters involved (Fig 3B). Regarding soil characteristics, the pH (11%) and the clay content (5.8%) were the main drivers of bacterial richness, with pH

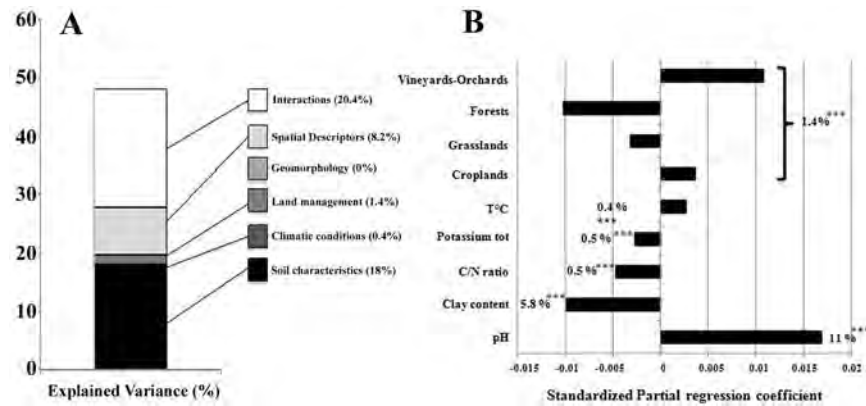


Fig 3. Variance partitioning, contribution and effect of model parameters for the distribution of bacterial richness on the scale of France. (A) Variance partitioning of bacterial richness. The amount of explained variance corresponds to the adjusted R^2 values of the contextual groups using partial redundancy analysis. The significance level of the contribution of the sets of variables is at least $P < 0.01$. (B) Model parameters for the distribution of bacterial richness on the scale of France. Each parameter is presented with its estimated model coefficients and its marginal effect assessed by a permutation test. $P < 0.01$: **, $P < 0.001$: ***. Missing values indicate that the variable was not retained in the model. Sand was removed prior to model evaluation since it was represented by the opposite of the sum of silt and clay contents.

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having a positive effect (indicated by a positive sign for the standardized coefficient) conversely to the other parameters (Fig 3B). These results confirmed the overriding effect of pH as a stimulating factor of bacterial community diversity at various spatial scales [15,49,55]. The significant but negative effect of clay content might be partly explained by the decrease in heterogeneity at a microscale with increasing clay content, leading to a lower diversity of microbial habitats and thus to a smaller hosting capacity for various indigenous bacterial species [56]. In addition, the C:N ratio as well as total Potassium content had a weak (0.5%) but significant negative effect on bacterial richness distribution, (Fig 3B). This was consistent with several reports highlighting that soils with a high C:N ratio, corresponding to a high recalcitrance of soil organic matter to degradation by microbes harboured a lower richness of microorganisms [57,58]. The weak (0.4%) but positive influence of climate (temperature in $^{\circ}\text{C}$) and geomorphology on bacterial richness was in agreement with other reports observing that these distal filters were little involved in microbial abundance and diversity distribution [12,23]. On the other hand, at larger scales, like continental or global scales with wider ranges of parameters (e.g. range: 2.5 to 25.7 $^{\circ}\text{C}$ for mean annual temperature for Zhou et al., (2016)), the temperature can influence microbial diversity distribution (directly, or indirectly by impacting plant cover), or the distribution of specific groups like the *Cyanobacteria* [14]. Altogether, these results modulate the hypothesis that the main filters driving the biodiversity of macro- and micro-organisms are different [24,51,59].

Independently of the other environmental variables, on this scale land use accounted for a small proportion (1.4%) of the explained variance, in agreement with previous reports that bacterial richness is generally poorly impacted by land use [2,60]. Even if both soil properties and land use have discriminating effects on soil bacterial diversity, cross-effects may have occurred, since soil pH and organic carbon content, for example, could also be dependent on land use and especially on agricultural practices (liming and tillage, respectively; [49]). On the other hand, less fertile soils (acidic, sandy) have been historically dedicated to forests [23]. In addition, by comparing the signs and values of the standardized estimated coefficients of land use categories, we demonstrated that bacterial richness was negatively impacted by natural or semi natural land uses *i.e.* forests and grasslands, and positively by perennial (vineyards-

orchards) or annual crop systems (Fig 3B). This observation supported a positive relationship between bacterial richness and soil disturbance due to cropping intensity, with Vineyards-Orchards > Crops > Grasslands > Forests, combining different types of agricultural practices (tillage, crop protection, fertilization, crop rotation; [61]). According to the "humped-back" model describing the response of the diversity of a community to environmental stress [62], a decrease in apparent diversity may occur (i) in a highly stressed environment due to dominance of particularly competitive species through selection, and (ii) in a notably unstressed environment, due to the dominance of particularly adapted species through competitive exclusion [63]. Contrastingly, moderate stress may increase apparent diversity, due to a diminution in competitive niche exclusion and in selection mechanisms. Our results showed that soils under annual (croplands) or perennial crop systems (vineyards/orchards) would correspond to these conditions, as they harboured highest richness levels compared to forests and grasslands (considered as unstressed environments) [3,61].

The spatial descriptors of the studied area, illustrating neighbourhood relationships between samples, corresponded to 26 significant (Principal Coordinates of a Neighbour Matrix), each representing different spatial scales (coarse: 110 to 250km, medium: 60 to 110km and fine: 30 to 60km, Table 2). The whole variance of bacterial richness explained by spatial descriptors was 8.2% and ranged from 0.19% to 0.74% according to PCNM. This scale dependency may reflect the effect of unmeasured spatial gradients [52], but may also be related to dispersal limitation of bacterial communities in regards of the large number of explanatory variables introduced in the analysis [54,64]. The influence of the scale was ranked by comparing the signs of the standardized coefficients and by cumulating the explained variance for each scale. A larger number of PCNMs (17) describing the fine scale were involved in explaining bacterial richness, with 5.25% of the cumulated explained variance, whereas those representing the coarse and medium scales were fewer (2 and 7 PCNMs with 1.02% and 1.89% of the cumulated explained variance, respectively). At fine and medium scales, the influence of spatial descriptors might be partly related to variations in unmeasured soil characteristics and land use, whereas at coarse scale it might result from geomorphology or the distribution of overall land cover (forest, grassland, mountains, sea). Our observation suggests that landscape configuration would be a significant driver of soil bacterial richness as also demonstrated on biodiversity turnover [24]. In addition, our analysis revealed numerous negative effects of spatial descriptors on bacterial richness at medium and fine scales, thus confirming that landscape configuration would be a significant driver and might partly affect bacterial richness by limiting bacterial dispersal as also demonstrated previously [24]. Altogether, our results showed that biogeographical patterns of bacterial richness can be explained by both selection (*i.e.* environmental filters like pH or C:N) and neutral processes (*i.e.* dispersal limitation), each being non-exclusive.

Predictive model of soil bacterial richness

Based on the RMQS dataset of bacterial richness and environmental parameters we have developed a predictive statistical model to provide a reference value of bacterial richness for a given pedoclimatic condition. The linear models with the smallest BIC (-700) and the largest R^2_{adj} (0.34) highlighted eight environmental parameters as significant explanatory variables of the bacterial richness, which were ranked as follows: pH > Clay content > C:N ratio > X (longitude) > elevation > C_{org} content > Y (latitude) > Silt content (Fig 4). This observation confirmed and refined the hierarchy of the environmental filters obtained with variance partitioning (Fig 3). At this step, climate data are not retained despite their significant role, since they are rarely available and expensive to obtain, limiting the use of this model to

Table 2. Model parameters of spatial descriptors for the distribution of bacterial richness on the scale of France. Considering that the major part of the environmental selection was measured by the previous explanatory variables (soil properties, land-use, etc.), we investigated the effect of dispersal on the residuals of the variance partitioning models. To do that, the neighbourhood between sites at various classes of distance was evaluated, using a Principal Coordinates of Neighbour Matrix approach (PCNM). Each spatial descriptor is presented with its estimated model coefficients and its marginal effect assessed by a permutation test ($P < 0.05$). Missing values indicate that the variable was not retained in the model. Spatial components were summarized according to the spatial scale considered: coarse, medium or fine.

Scale	Spatial descriptors (PCNM)	Explained variance (%)	Model coefficient
Coarse [110km; 250km]	PCNM ₁₃	0.28	0.19
	PCNM ₂₉	0.74	-0.29
Medium [60km; 110km]	PCNM ₅₈	0.30	0.19
	PCNM ₇₅	0.28	-0.19
	PCNM ₁₁₃	0.25	-0.18
	PCNM ₁₂₀	0.23	-0.17
	PCNM ₁₂₆	0.38	0.21
	PCNM ₁₂₈	0.19	0.16
	PCNM ₁₄₂	0.26	0.18
Fine [30km; 60km]	PCNM ₁₈₈	0.47	-0.23
	PCNM ₂₁₁	0.25	0.18
	PCNM ₂₁₆	0.29	-0.19
	PCNM ₂₆₄	0.56	-0.25
	PCNM ₂₇₅	0.33	-0.20
	PCNM ₂₈₁	0.19	0.16
	PCNM ₂₉₆	0.22	-0.17
	PCNM ₃₀₅	0.26	-0.18
	PCNM ₃₁₆	0.27	-0.18
	PCNM ₃₁₉	0.43	-0.23
	PCNM ₃₂₇	0.46	-0.23
	PCNM ₃₅₉	0.23	-0.17
	PCNM ₃₈₇	0.20	0.16
	PCNM ₄₂₆	0.19	-0.16
	PCNM ₄₂₇	0.26	0.18
	PCNM ₄₃₆	0.35	-0.21
PCNM ₄₆₆	0.29	0.19	

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compare predicted and measured values in a context of soil biological diagnosis for and by soil users [17]. Alternatively, climatic conditions were replaced in the model by longitude, which in France is integrative of climate and soil moisture and did not reduce the R^2 of the model and therefore its robustness [25].

First, we developed a linear polynomial regression model based only on pH as explanatory variable, as previously described [12]. By testing the increasing complexity (from simple linear and up to the fifth degree) of the polynomial model, we found that the model degree four gave the best R^2 (0.34) since at higher degree the R^2 remained virtually unchanged (Fig 5). Nevertheless, in their study based on 100 soils, Fierer & Jackson, 2006 obtained a higher R^2 (0.58) with a degree 2. This difference might be partly explained by our deeper sampling effort, but mostly by the genotyping technics used by these authors (T-RFLP), which could limit the variability of the estimated richness (several tens of populations) by comparison with pyrosequencing technology (several tens of thousands of OTU in our case), as previously shown [4]. To improve the model in terms of higher R^2_{adj} and to get closer to the normality hypotheses and to improve the variance homogeneity of the residuals [65], we tested for its ability to include interactions between explanatory variables identified [17,65,66]. In addition to pH, we selected

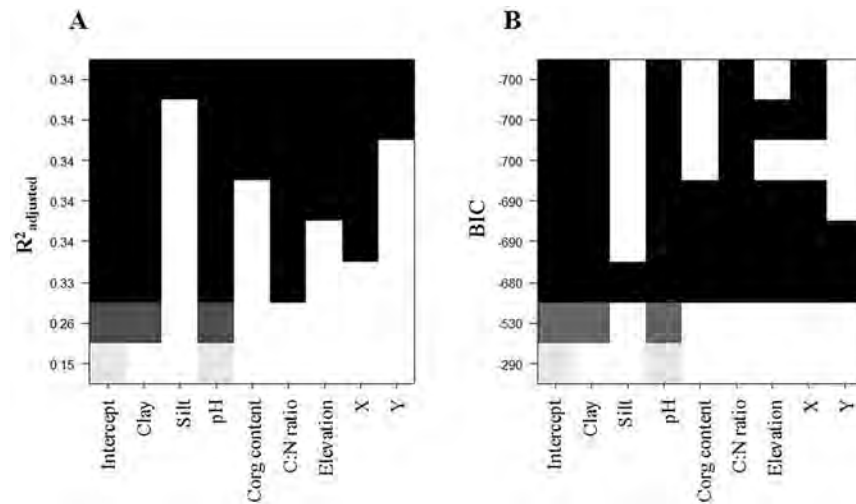


Fig 4. Hierarchy of the linear models of soil bacterial richness involving soil physicochemical, geographical coordinates and climatic variables. The hierarchy of the linear models implying environmental variables is given according to the R^2_{adj} criterion (A) and the BIC criterion (B) with the exhaustive method. Each row in this graph represents a specific model. The variables included in a given model are represented by means of shaded rectangles. The intensity of the shading represents the ordering of the BIC and R^2_{adj} values according to the absolute value.

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only three main explanatory variables to be included in the model: Clay content, C:N ratio and longitude (X), since the R^2 of the model based only on pH was not significantly improved by integrating additional variables (data not shown). Finally, the model developed has a $R^2 = 0.56$ and a $R^2_{adj} = 0.58$ and the following mathematical form (all parameters of the models are given

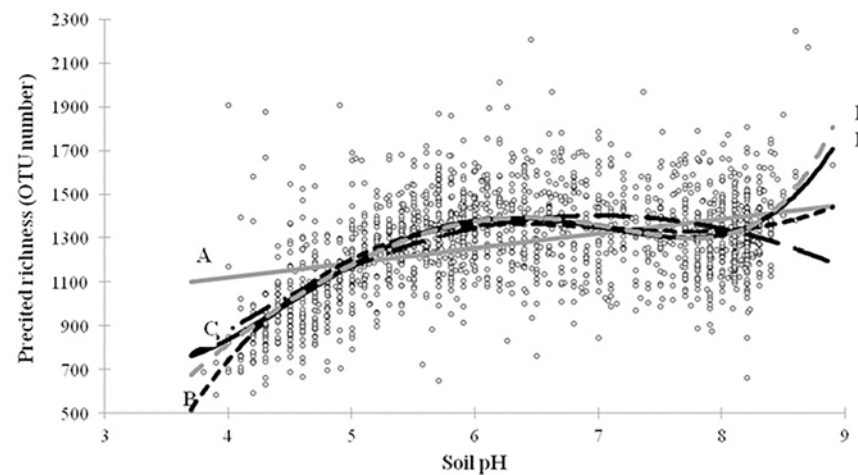


Fig 5. Polynomial regression between the bacterial richness and soil pH for different level of increasing complexity (from simple linear and up to the fifth degree) of the model. (A) Grey line, simple linear model, bacterial richness = $66.48 \cdot \text{pH} + 855.43$; $R^2 = 0.15$. (B) Dotted black curve, quadratic model, bacterial richness = $-58.55 \cdot \text{pH}^2 + 818.8 \cdot \text{pH} + 1460.6$; $R^2 = 0.29$. (C) Dotted black curve, cubic model, bacterial richness = $25.458 \cdot \text{pH}^3 + 537.9 \cdot \text{pH}^2 + 3751.5 \cdot \text{pH} + 7287.4$; $R^2 = 0.32$. (D) Black line, model degree four, bacterial richness = $13.06 \cdot \text{pH}^4 + 302.64 \cdot \text{pH}^3 + 2499.2 \cdot \text{pH}^2 + 8510.8 \cdot \text{pH} + 10919$; $R^2 = 0.34$. (E) Dotted grey curve, model degree five, bacterial richness = $2.63 \cdot \text{pH}^5 + 69.59 \cdot \text{pH}^4 + 720.07 \cdot \text{pH}^3 + 3731.7 \cdot \text{pH}^2 + 10171 \cdot \text{pH} + 11127$; $R^2 = 0.34$.

<https://doi.org/10.1371/journal.pone.0186766.g005>

in the [S2 Table](#)):

$$\begin{aligned} \text{Richness} = & 1044 + 3.305 * \text{pH}^4 - 0.0457 * \text{Clay}^2 + 0.0597 + 0.00298 * \text{Clay}^2 * C \\ & : N - 1.54 * 10^{-6} * \text{Clay}^3 * C : N + 2.336 * 10^{-5} * (C : N)^2 * X. \end{aligned}$$

To validate the model, we evidenced the normality distribution of model residuals, which was confirmed by the Shapiro-Wilk test of normality ($P = 0.149$), as well as a good homogeneity of the residuals tested by the Breush-Pagan test of homogeneity ($P = 0.5469$). In addition, plotting the measured richness against the predicted richness using the cross-validation dataset revealed an important scatter of the points around the $y = x$ line ([S3 Fig](#)), which validated the high predictive ability of the developed polynomial model. Finally, the sensitivity of the model to measurement errors on each explanatory variable was evaluated by a sensitivity index [17]. This analysis demonstrated that the model was highly sensitive to variations in pH, clay content and C:N ratio, together with their interactions and cubic effects, which was in agreement with the above discussion concerning the variance partitioning analysis ([S3 Table](#)). However, one limitation of our model is the absence of variations in bacterial richness between different years (or between seasons during one year) that can result from modifications of climatic conditions, plant cover or agricultural practices. In complement to our study, it will be relevant to validate the robustness of this model on a sampling strategy that integrates such temporal variation.

Altogether, our study provides the first French national atlas of soil bacterial richness using an extensive sampling survey of about 1,800 samples, and confirms the relevance of investigating microbial community on a nationwide scale to better understand the ecological processes involved in regulating microbial richness. We showed that the distribution of bacterial richness at this scale was heterogeneous and spatially structured, mainly driven by proximal filters such as soil characteristics and land use (both supporting a selection process) but also significantly influenced by spatial descriptors (potentially supporting dispersal limitation in microbial populations, derived from neutral theory, or the influence of unmeasured soil properties). This nationwide spatial scale was also shown to be relevant for evaluating overall land use in the context of a sustainable use of soil resources. Based on the referential dataset, the predictive model developed in this study complements the one developed for molecular microbial biomass [17], as both present innovative and operational mathematical tools for assessing a comprehensive soil microbiological status in the French pedoclimatic context. Comparison of predicted and measured values provides a robust diagnosis of soil microbial abundance and diversity and their evolution under environmental pressures such as agricultural practices, industrial pollutions or more global changes. Altogether, mapping and a predictive model of bacterial richness involving over 1,700 geo-located samples covering the French territory could help policy makers to produce conservation policies based on soil biodiversity. Based on this primary analysis of bacterial richness other aspects of soil bacterial beta-diversity such as evenness, community structure, taxa-area relationships and variations in the core bacterial taxa across France need to be investigated to have a comprehensive overview of the biogeography of microorganisms.

Supporting information

S1 Fig. Distribution of detected bacterial OTU numbers in French soils. The curves correspond to simulation of normal distributions (dotted line with estimated parameters: average: 1288.53 ± 207.39 for OTU number) and log normal distributions (black line with estimated parameters: average: 7.1471 ± 0.1719 for OTU number). Normal and log normal distributions

were obtained using Maximum Likelihood estimations.
(PDF)

S2 Fig. Cumulative curve of different detected OTUs according to the number of studied soils. The thickness of the curve represents the standard deviation obtained from 1,000 cumulative curves with a random selection of soils.

(PDF)

S3 Fig. Relationship between the measured and the predicted values of bacterial richness by applying the polynomial model of degree four on the cross validation dataset (180 soil samples). The black line represents the 1:1 line ($y = x$).

(PDF)

S1 Table. Bioinformatic parameters and databases used in the analysis of 16S rRNA gene sequences.

(DOCX)

S2 Table. Summary of model coefficients and significance. This table describes the Coefficients $\hat{\beta}$ of the fourth degree polynomial model for each of its components. The standard error of each coefficient and its significance is also provided ($P < 0.05$).

(DOCX)

S3 Table. Overview of the model sensitivity analysis. The Standardized Regression Coefficients (SRC) of the variables to which the model is most sensitive are presented here. The variables are organized according to the absolute value of their associated SRC from the highest to the lowest.

(DOCX)

S1 File. Influence of the normalization step on RMQS sample representativeness. 100 replicates of the normalization step were done on each of the 200 randomly selected samples (10% of the samples). For each replicate, high-quality-reads were clustered, and obtained OTUs analyzed to determine the impact of the normalization step on OTUs. Four groups of OTUs were considered: Major (composed of more than 1% of reads), Medium ($1 \pm 0.1\%$ of reads), Low ($0.01 \pm 0.1\%$ of reads) and Rare (less than 0.01% of reads), showing no impact of the normalization step.

(ZIP)

S2 File. Rarefaction curves of RMQS samples computed after normalization step.

(ZIP)

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Microbial diversity and ecological networks as indicators of environmental quality

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Abstract Evaluating the quality of ecosystems in terms of biological patrimony and functioning is of critical importance in the actual context of intensified human activities. Microbial diversity is commonly used as a bioindicator of ecosystems functioning. However, there is a lack of sensitivity of microbial diversity indicators in the case of moderate and chronic environmental degradation, such as atmospheric deposition of pollutants, agricultural practices, diffuse pollution by wastewater and climate change. As a consequence, there is a need for alternative bioindicators of soils and water quality. Here, we discuss the interest of adopting a more integrative approach based on biotic interaction networks beyond the simple diversity indicators. We review how the various biotic interactions can be integrated in the various microbial networks such as trophic, mutualistic and co-occurrence networks. Then we discuss the efficiency of microbial networks and associated metrics to detect changes in microbial communities. We conclude that the connectance, the number of links and the average degree of co-occurrence networks could vary from 10 to 50% in response to minor perturbations when microbial diversity parameters remain stable. Finally, we analyze studies that aimed at linking microbial networks and activity to evaluate the potential of such networks for providing simple and operational indicators of ecosystem quality and functioning.

Keywords Microbial interactions · Taxonomic diversity · Co-occurrence network · Environmental quality · Indicators · Diagnosis

Introduction

Intensification of human activities over the past 60 years of the anthropocene has led to more rapid and extensive changes in ecosystem structure and functioning than throughout human history as a whole (Millennium Ecosystem Assessment 2005; Regnier et al. 2013; Steffen et al. 2007). In this environmental “state of emergency”, it becomes urgent to have sensitive and robust indicators for identifying, measuring and monitoring the modifications of ecosystems in response to these perturbations. Among the physical, chemical and biological components of ecosystems, the biological component is particularly sensitive to perturbations. Sala (2000) demonstrated that up to 50% of global biodiversity can be modified and ranked the principal causes as follows: land use > climate > air pollution > greenhouse gas emission. Within the biological component, micro-organisms constitute a major part of the genetic patrimony, due to their ubiquity, abundance and their genetic and metabolic diversities, and are the principal actors in the biogeochemical processes (Fitter et al. 2005; Nielsen et al. 2011). In addition, their small size, short generation time and genetic plasticity confer them with a capacity for rapid adaptation to changes in the environment. For all these reasons, they are very good bioindicators of the impact of environmental perturbations and of ecosystems quality (Bouchez et al. 2016).

The historically used microbial indicators are microbial biomass and measurements of activities, such as respiration, catabolic capacities, recycling of mineral elements,

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degradation of pollutants (Ritz et al. 2009). More recently, indicators of taxonomic and functional diversities have been developed thanks to advances in the field of molecular biology (Bouchez et al. 2016). The many studies carried out on these indicators have provided sufficient hindsight regarding their sensitivity, robustness and specificity to be aware of their limitations. Most of the current studies involving these diagnosis tools are concerned with the impact of high-intensity disturbances, which increase the probability of responses by microbial indicators. However, some studies of moderate and chronic, single or multiple, perturbations have shown that these indicators of microbial taxonomic diversity are not always very sensitive to detect early modifications (Ager et al. 2010). As the need to emphasize significant effects can often prevent the publication of research work, there are few examples in the literature. Nevertheless, it has recently been reported that the diversity of microbial communities associated with terrestrial bryophytes is not sensitive to chronic atmospheric pollution with particles and gases emissions below the thresholds of World Health Organization (Karimi et al. 2016). These indicators might therefore lead to the conclusion that the ecosystem remains unaltered if the perturbation is of intermediate intensity. In certain cases, indicators of microbial diversity can also respond in an unexpected way. It was shown that soil contamination with mercury for several decades did not affect the diversity of fungal communities but increased those of bacteria (Zappellini et al. 2015). In addition, mechanical disturbance of the soil such as tillage seems to increase bacterial diversity, whereas a reduction in this diversity might have been predicted, due to destruction of the soil micro-habitats (Lienhard et al. 2014). These limitations and unexpected results are providing a framework for the development of new finer-tuned indicators that are better adapted to environmental diagnosis in the current context where many environmental perturbations are weak, complex and chronic over the long term.

A multi-dimensional and more integrated vision of ecosystems and their organization is provided by networks of biotic interactions. These networks are intimately linked to ecosystem functioning and play a crucial role in maintaining biodiversity (Vinebrooke et al. 2004; Wardle 2006). These relationships have been well illustrated in some studies on macro-organisms in the context of man-induced perturbations. Geslin et al. (2013) showed that the network of interactions occurring between plants and their pollinators exhibited a profound variation in structure along the gradient from semi-natural, farming, suburban to urban environments. Despite recent important methodological advances in characterization of the microbial communities in environmental matrices, microbial interactions networks have been little studied and are not yet used as indicators of

the functioning and state of an ecosystem when subjected to perturbation. However, in view of the reactivity and diversity of these microbial interactions, and the enormous pool of genetic and functional diversity characterizing the microbial world, such networks could provide a precious source of information about the ecological state of ecosystems.

In this review, we demonstrate the interest of changing the focus on the microbial compartment of ecosystem from a taxonomic diversity point of view to an “interactions networks” approach to gain a more integrated vision of the relationships between *taxa*. Among the microbial interaction networks currently the most studied, different types of networks can be distinguished, depending on whether they are based on a single type of relationship, e.g. trophic and mutualistic, or on the resulting complex interactions, e.g. co-occurrence networks. In the first section, we review the different types of interactions occurring between micro-organisms in ecosystems which have been used to define the different types of networks. In the second section we focus on co-occurrence networks, which are the most comprehensive and simplest to implement. We then examine existing studies on the application of such networks to microbial communities and summarize their response to environmental perturbations. Thus, we determine the potential of microbial co-occurrence networks as bioindicators of terrestrial ecosystems quality and as tool to complement and compensate for the reported lack of sensitivity or specificity of taxonomic diversity indicators.

From biotic interactions to interaction networks

Biotic interactions in microbial communities

During the course of evolution, the emergence of species and their coexistence in space and time has led to the establishment of multiple interactions. In fact, interactions such as metabolism and reproduction appear to be one of the fundamental traits of life and take on particularly diverse forms in micro-organisms (Bertrand et al. 2011). Interactions can be beneficial, antagonistic or neutral regarding the impact they have on the species involved (Lidicker 1979) (Fig. 1).

Beneficial interactions

Commensalism is the only beneficial interaction which benefits just one of the two partners. In commensal relationships, one partner benefits from the interaction but does not cause losses to the other partner. It is often illustrated by biodegradation, which corresponds to the consumption by commensal organisms of compounds produced by other

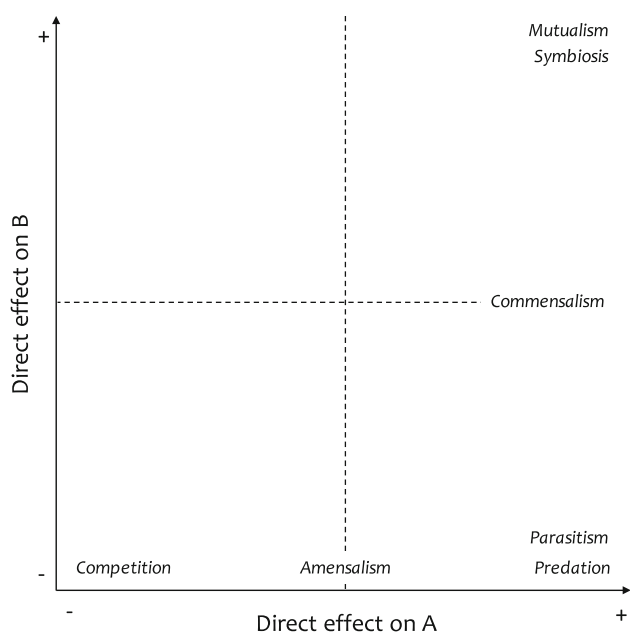


Fig. 1 Schematic representation of the biotic interactions and their direct effect on micro-organisms. – negative effect on micro-organisms’ fitness; +: positive effect on micro-organisms’ fitness

members of the community. The commensal biodegradation of cellulose, a complex organic compound present in numerous natural environments, is one such example (Soundar and Chandra 1987; Kato et al. 2005).

Mutualism is a win–win situation in which both species profit from the interaction. The genetic distance between species is not a hindrance to the partnership, and mutualistic interactions can occur between plants and animals, between bacteria and vertebrates, or between micro-organisms and plants. *Symbiosis* is a special case of mutualism in which the interaction is indispensable to the maintenance and growth of at least one of the two partners. One of the most well-known examples of *symbiosis* is the association between nitrogen-fixing bacteria and/or mycorrhizal fungi and plants (van der Heijden et al. 2008). These micro-organisms, due to their role in biogeochemical cycles, are, respectively, responsible for up to 80% of the nitrogen acquired by plants each year and more than 75% of the phosphorus, which can lead to an increase in plant productivity as high as 50% (van der Heijden et al. 2008). In addition, microbial *mutualism* can sometimes involve trophic aspects, detoxification and toxin synthesis. This is the case of *syntrophy*, an interaction in which the association of micro-organisms with complementary metabolisms facilitates their access to trophic resources. For example, *Synthropsus aciditrophicus* (*Deltaproteobacteria*) is able to metabolize various fatty acids and aromatic acids under anaerobic conditions, but only if hydrogenotrophic bacteria are present in the environment (Bertrand et al. 2011). This phenomenon is also observed during the

degradation of xenobiotics where several species “help” to metabolize a complex molecule into several simpler by-products. For example, the degradation of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) involves two species of fungi and a bacterial species which break down the molecule into at least five by-products (Subba-Rao and Alexander 1985).

Antagonistic interactions

The most widespread antagonistic interactions are the *predator–prey* (*predation*) relationships. Taking all ecosystems together, these biotic interactions have received the most study. In terrestrial microbial ecosystems, the most thoroughly investigated and well-known antagonistic interactions concern the *predation* of bacteria and fungi by amoebas and nematodes (Gilbert et al. 1998; Rosenberg et al. 2009; Bonkowski and Clarholm 2012). Amoebas are protozoans (single-celled organisms), which have a very varied diet and variable trophic levels. Their prey can be bacteria, micro-algae, cyanobacteria, fungi in hyphal or spore form, other amoebas, ciliates and even micro-metazoans such as nematodes (Jassey et al. 2013). As a whole, predation relationships form the microbial loop in ecosystems. In soils, this loop results in nitrogen release, which improves biological fertility for plants.

The *host–parasite* relationship differs from predation in that the parasite is physically associated with its host for at least part of its biological cycle, whereas the predator remains free-living. The exchanges of signals at the molecular level are therefore spread over much longer periods involving the host’s defence system and the parasites strategies of getting around these defences. For example, *Fusarium*, a phytopathogenic fungus of a large number of plant species, causes a yield loss of 30–99% in chickpea (Navas-Cortés et al. 2000) and 45–100% in tomato (McGovern 2015), depending on the virulence of the *Fusarium* strain and susceptibility of the cultivars. Other cases of parasitism are known between fungi and bacteria, such as the fungus *Agaricus bisporus* attacked by *Pseudomonas tolaasi* (Wells et al. 1996).

Two types of antagonistic interactions are not win–lose relationships. *Amensalism* is an interaction in which one partner is adversely affected without conferring an advantage to the other partner. It is based on a physical or chemical modification of the environment leading to the release of toxic compounds or secondary metabolites from one species, metabolites which alter the environment to the detriment of the other micro-organisms (this is the case of some bacteria which reduce the pH of the environment). *Competition* between organisms is a lose–lose relationship in which both partners use the same resource, whether this is a nutrient, water or space. The partners involved have

very similar needs and use comparable, if not identical, means to acquire their resources. Competition can occur between species of equivalent trophic levels or between organisms at different trophic levels, even between different kingdoms. One of the oldest illustrations of competition is the famous experiment by G.F. Gause in 1934 who, after placing two species of paramecia *P. aurelia* and *P. caudatum* in co-culture, formulated the law of “competitive exclusion” for two species with similar ecological niches (Gause and Witt 1935). In soil, bacteria and fungi compete in the decomposition of simple organic compounds, e.g. root exudates, or more complex ones, e.g. plant tissues. This competition is sometimes expressed by antibiosis, i.e. the emission of antibiotic by one of the competitors, a mechanism exploited in biological control. For example, this is the case of *Pseudomonas fluorescens*, a bacterium used in the biocontrol of *Fusarium*, a pathogenic fungus of several plant species (Schouten et al. 2004).

Networks of interactions

Organisms, particularly micro-organisms, are not isolated within ecosystems and this is the same for biotic interactions since one organism can be involved in many different interactions with multiple other organisms. All these interactions can occur simultaneously in time and space, and constitute altogether a network of interactions. To make the interaction networks easier to understand, they are usually represented by just one type of relationship. Thus, the main types of networks distinguished have been trophic networks, also called food webs, and mutualist networks.

Trophic networks

Trophic networks have been very widely studied and refer solely to the feeding relationships between the consumers and their resource(s). A given consumer can have several resources and a resource can have several consumers. A trophic network consists of a tangle of trophic chains with primary producers or detritivores, namely bacteria and fungi, at the base, followed by 3–5 heterotrophic levels: a level of secondary consumers which consume the primary producers or the detritivores, several levels of predators which feed on the secondary consumers and the other predators, and a level of predators at the top of the chain (Neutel et al. 2002).

The different levels of consumers along this trophic chain are able to feed on all the lower trophic levels. As shown in Fig. 2, nematodes have a varied range of resources and can be bacterivores, fungivores, phytophages, predators or omnivores. In addition, the nutritive resources of some micro-organisms depend on their

physiological state caused by the environmental conditions. For example, *Hyalosphenia papilio*, a mixotrophic testate amoeba found in peatlands, adapts its metabolism and diet as a function of the prey availability by reversely changing its behaviour from predator to primary producer (symbiotic photosynthesis) (Jassey et al. 2013). This mixotrophic ability is known in a large number of protozoans, such as amoebae and ciliates.

At the microbial scale, these interactions are regarded as the flux of carbon and nitrogen between different compartments and are used to measure matter and energy transfers in biogeochemical cycles (Ducklow 1994; Davidson and Janssens 2006). It should nevertheless be noted that the examination of these trophic networks is strongly dependent on modelling to determine coefficients of assimilation between the different compartments. This makes microbial trophic networks difficult to evaluate, mainly theoretical, and hard to interpret in simply. Therefore, these networks are not particularly operational and difficult to apply as bioindicators of ecosystem quality. At present, their use is essentially limited to theoretical research on trophic relationships and balances of ecosystems.

Mutualistic networks

Mutualistic networks have been little studied at the microbial scale and those existing between plants and pollinating insects are better known (Bersier 2007). One of the most important mutualistic microbial networks is biofilm, which can be defined as an agglomeration of micro-organisms, for example bacteria, fungi, micro-algae, amoebas and nematodes, embedded in a matrix of biopolymers and developing on the surface of a support. Biofilms were formed by the flow of a non-sterile liquid on a surface. The bacteria from the liquid progressively attach themselves to the surface and synthesize biopolymers, mainly sugars and proteins, which are excreted in order to protect the bacteria and strengthen their adhesion (Davey and Toole 2000). Cooperation between the different biofilm protagonists helps to extend and consolidate the protective matrix, and therefore to improve the defence of the contributors as a whole. The mechanisms of interactions within this multi-specific network are still poorly understood. Nevertheless, a molecular communication mechanism between the organisms has recently been described (known as quorum sensing), which may facilitate biofilm coordination (Ross-Gillespie and Kümmerli 2014).

Co-occurrence networks

Studying networks based on a single type of relationship provides a simplified idea of communities and a better

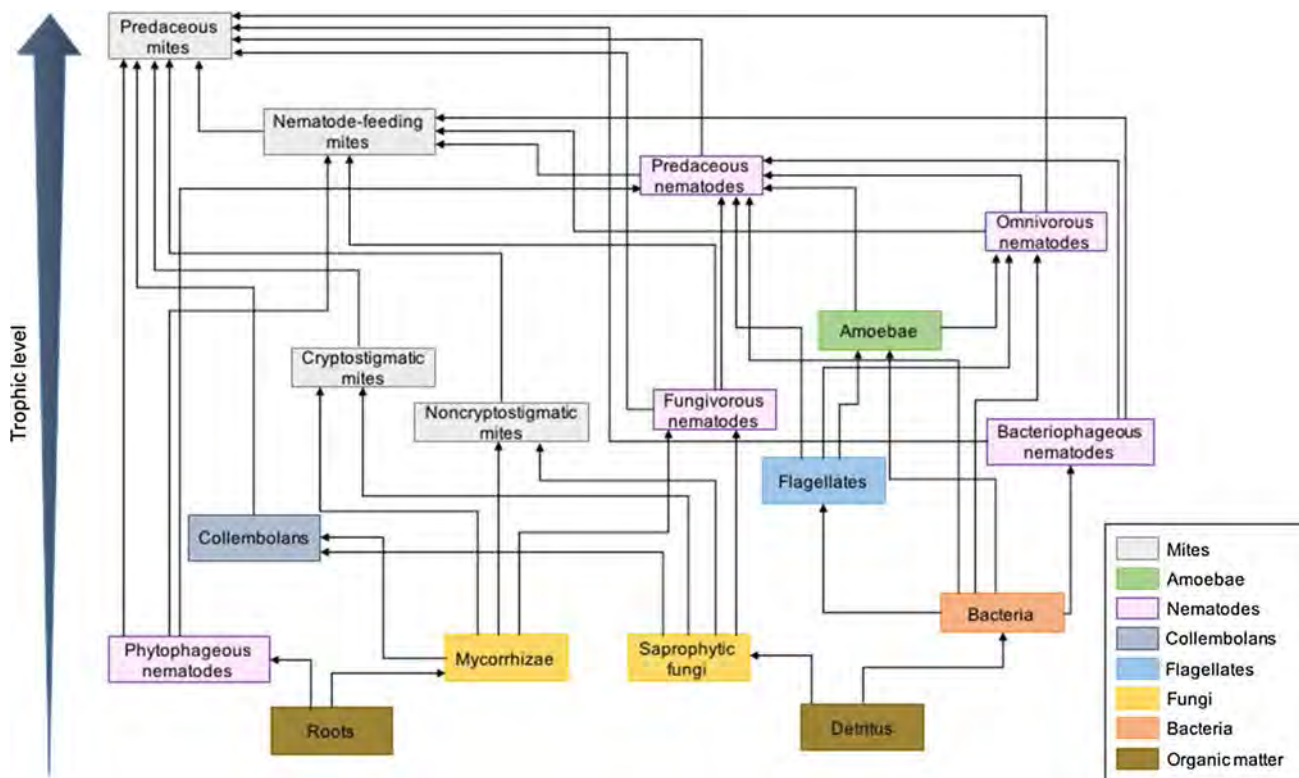


Fig. 2 Example of a complex microbial trophic network in soil (Neutel et al. 2002). Arrow represents the feeding relationship between microbial groups and sources of nutrients in the network

understanding of their organization. However, these networks do not integrate the true complexity of ecosystems and their analysis can lead to a partial, even biased image of the state of an ecosystem.

Unlike trophic and mutualistic networks, co-occurrence networks provide an integrated vision of all the relationships existing between micro-organisms in a given environmental matrix. Two microbial species can interact simultaneously in numerous ways, whether by direct routes (as described in part I.1.) or indirectly. Like direct interactions, indirect biotic interactions can be involved in maintaining species diversity. Examples can be cited of interactions based on the expression of complex metabolic pathways (Levy and Borenstein 2013; Zelezniak et al. 2015), or the effect of engineering species (Jones et al. 1994), i.e. organisms which modulate, either directly or indirectly, the availability of resources or the quality of the habitat for other species by causing changes in the physical and chemical state of the environment. The combined result of all these interactions can lead to joint evolution of the organisms in the environment (positive relationship known as co-occurrence), their opposite evolution (negative relationship known as co-exclusion) or to the absence of a relationship (this imply whether a true lack of interaction or a null combination of all interactions between the organisms) (Gross 2008). At the community scale, these

positive or negative relationships together constitute a co-occurrence network.

Historically, the study of co-occurrence networks was first initiated by J.M. Diamond in 1975. By examining communities of birds in New Guinea, he showed that the assembly rules in species which included competitive interactions rose to non-random patterns of co-occurrence (Diamond 1975). Until the year 2000, co-occurrence networks were the subjected to great controversy. These were mainly led by Connor and Simberloff, who demonstrated that the patterns previously associated with competition by Diamond could also result from random colonization (Connor and Simberloff 1979). In 2000, Gotelli and McCabe subjected 96 data sets to meta-analysis and confirmed that the co-occurrence approach effectively reflected the non-randomness of community assemblages. However, they concluded that the patterns obtained could not be explained by competitive mechanisms alone (Gotelli and McCabe 2002), thus bringing 25 years of debate to an end.

Co-occurrence networks reappeared in the literature only in 2010 and most investigations involving this approach were then focused on microbial communities. In fact, most of the interactions occurring in microbial communities cannot be individually observed, identified or quantified due to the size of the organisms and the

complexity of environmental matrices. Nevertheless, all these interactions can be integrated by the co-occurrence network approach and quantified with the same molecular tools that are used to measure taxonomic diversity. Studies of co-occurrence networks in microbial communities have been carried out in marine environments (Fuhrman and Steele 2008; Steele et al. 2011; Røling et al. 2014), soil (Barberán et al. 2011; Lentendu et al. 2014; Zappelini et al. 2015; Sauvadet et al. 2016), sediments (Bissett et al. 2013), the human body (Faust et al. 2012) and the plant microbiome (Karimi et al. 2016; Van Der Heijden and Hartmann 2016). Most of these studies are based on inventories of the taxonomic diversity of communities obtained by high-throughput sequencing and to a lesser extent from counts and morphological differentiation observed by microscope (Sauvadet et al. 2016; Karimi et al. 2016).

According to the graph theory, a given co-occurrence network is constituted of nodes and links. Nodes, i.e. objects at the ends of the lines, represent the *taxa*, i.e. OTU, species, genera, families, classes, *phyla* or kingdoms or functional groups. Links, i.e. the edges between nodes, represent the significant relationships identified by statistical analysis (Table 1; Fig. 3). Mathematical methods for calculating co-occurrence relationships in microbial communities are still rapidly evolving towards statistical methods being increasingly robust to evaluate co-occurrence relationships and resulting in numerous methods available in the literature in recent years. At present, however, there is a lack of consensus and the choice of method most often depends on the characteristics of the data set, such as type of information, number of replicates, number of variables, independency of the variables and distribution of the variables

Although the statistical methods for identifying co-occurrence relationships are increasingly robust, very few investigations are being focused on comparing network architecture in control and disturbed situations. It is only very recently that such questions have been addressed (Williams et al. 2014; Blouin et al. 2015; Zappelini et al. 2015; Sauvadet et al. 2016; Karimi et al. 2016), and a more complex network was usually observed to be associated with the least perturbed situation (Blouin et al. 2015; Zappelini et al. 2015; Karimi et al. 2016). Disregarding the statistical method used for computing them, networks can be described through different metrics to characterize their architecture. Some metrics are centred on the behaviour of *taxa* and provide information about the diversity and type of relationships that each one establishes within the community while others indicate the overall organization of the network and provide information about the complexity of the microbial community within the ecosystem.

Metrics at the taxon scale

Several types of information are accessible at the *taxon* level (Fig. 4a):

- a *taxon* can be present without being connected. If connection is null, this means that its abundance is independent from that of the other *taxa*, even if interactions take place between them;
- if the *taxon* is connected, the number of direct linkages that it establishes with other *taxa* tells us about its first degree connections, i.e. the number of other *taxa* for which the abundance or biomass will evolve simultaneously with it. This is an indicator of complexity at the *taxon* level;
- among these connections, the proportion of positive and negative links (P:N ratio) provides important information about the global behaviour of the *taxon*. The sign associated with the linkage tells us about the direction of the relationship (positive or negative) between two *taxa*;
- the betweenness centrality of the *taxa* indicates the number of the shortest paths that pass through it among the shortest paths existing between every pair of nodes. The betweenness centrality of a node is independent of its dominance within the community and, consequently, very abundant *taxa* can have positions of little importance and inversely for less abundant *taxa*. If the node exhibits maximum centrality within the network, it is identified as a keystone *taxon*, i.e. the principal *taxon* which maintains the network structure and therefore, potentially, the functioning of the ecosystem.

Metrics at the network scale

The metrics calculated at network scale provide information about the topology and organization of the system (Fig. 4b). Although many different metrics can be calculated, only six allow reasonable ecological interpretations and are used regularly:

- the number of nodes, which must be differentiated from the specific richness, indicates the number of connected *taxons*. It is also important to retain the information about *taxa* that are present but not involved in a co-occurrence relationship.
- the total number of links in the network and the proportion of positive and negative links provide information about the density of the interactions and their type;
- the connectance is the number of potential links which are realized. It measures the network complexity and can be interpreted as the observed organization level of

Table 1 Compilation of the main mathematical methods used in the literature to evaluate co-occurrence relationships in microbial communities indigenous from various environmental matrices

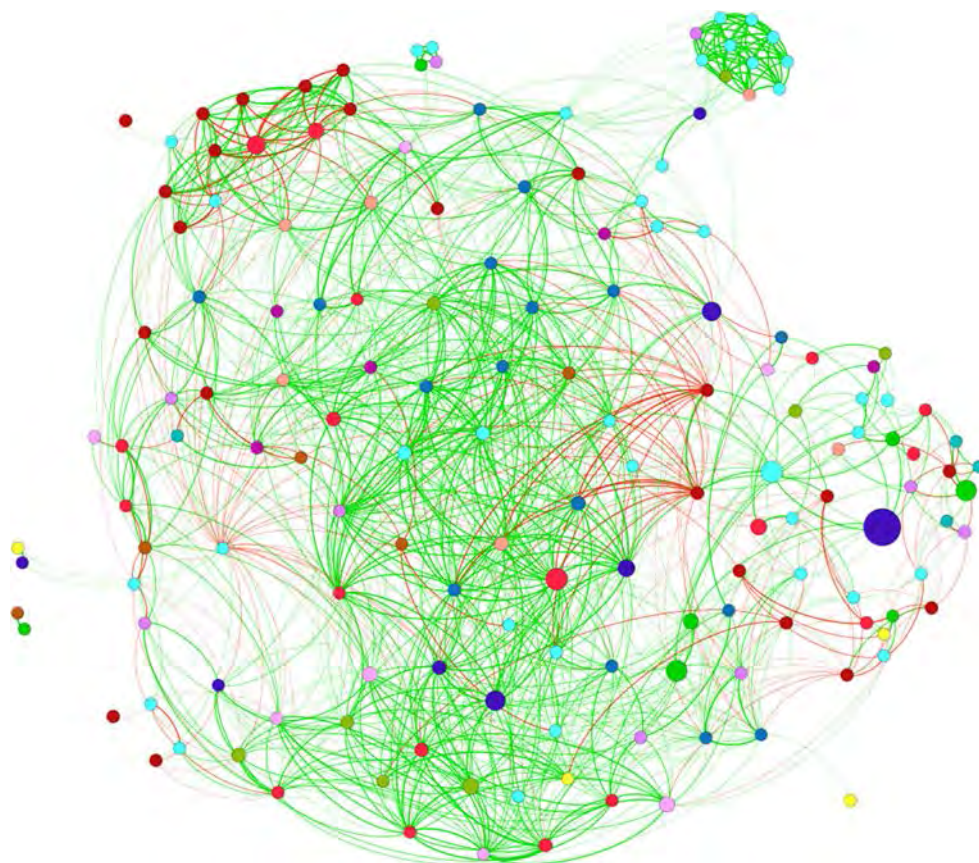
Method	Acronym	Description	References
Pearson correlation	PC	Abundance similarity	Faust and Raes (2012), Lupatini et al. (2014) and Zhou et al. (2011)
Spearman correlation	SC	Abundance similarity	Faust and Raes (2012), Williams et al. (2014), Kittelmann et al. (2013), Ma et al. (2016), Widder et al. (2014), Karimi et al. (2016) and Zappellini et al. (2015)
Euclidean distance	ED	Metric distance	Faust and Raes (2012)
Kullback–Leibler distance	KLD	Metric distance	Faust and Raes (2012)
Bray–Curtis distance	BC	Metric distance	Faust and Raes (2012)
Partial correlation		Abundance similarity	Faust and Raes (2012)
Generalized boosted linear model	GBLM	Regression	Faust and Raes (2012)
Sparse regression		Regression	Faust and Raes (2012)
PC + SC + KLD + BCD + GBLM		Combination of methods	Faust et al. (2012)
Draughtboard	C-board	Number of species pairs that never occur together	Diamond (1975), Horner-Devine et al. (2007) and Gotelli and Ulrich (2010)
Abundance Aggregation	AA	Count of pairs aggregation	Gotelli and Ulrich (2010)
Variance test of Schluter		Comparison of the variances of row totals with the sum of the column variances	Gotelli and Ulrich (2010)
Morisita index of similarity		Metric distance	Chao et al. (2008), Gotelli and Ulrich (2010)
Mantel test		Identification of non-randomness	Gotelli and Ulrich (2010)
C-score	C-score	Average number of draughtboard units between all possible species pairs in a matrix	Horner-Devine et al. (2007), Lentendu et al. (2014) and Gotelli and Ulrich (2010)
C-score + Spearman correlation		Combination of methods	Barberán et al. (2011)
Community structure index	Combo	Number of unique species combinations found between pairs of sites	Gotelli and McCabe (2002) and Horner-Devine et al. (2007)
Sparse correlations for compositional data	SparCC	Linear Pearson correlations between the log-transformed components	Friedman and Alm (2012), Berry and Widder (2014) and Milici et al. (2016)
Sparse Inverse Covariance estimation for Ecological Association Inference	SPIEC-EASI	Algorithms for sparse neighbourhood and inverse covariance selection	Kurtz et al. 2015
Local similarity analysis	LSA	Analogous to a Pearson/Spearman’s correlation	Steele et al. (2011) and Eiler et al. (2012)
Extended local similarity analysis	eLSA	LSA with replicates	Xia et al. (2011) and Chow et al. (2014)
Fisher’s exact test		Evaluation of co-occurrence for all possible combinations of 2, 3, and 4 species communities	Zelezniak et al. (2015)
Maximal information coefficient	MIC	Equals the coefficient of determination (R ²) of the data relative to the regression function	Reshef et al. (2011), Bissett et al. (2014) and Peura et al. (2015)
Cumulative hypergeometric <i>P</i> value			Freilich et al. (2010)
Non-metric multi-dimensional scaling	NMDS	Strength of interactions are presented by proximity	King et al. (2012)

network compared to its potential organization level. Certain authors refer to it as the interactions diversity. From an ecological viewpoint, it would reflect the incidence of ecosystemic processes where a greater complexity is interpreted as a more intense activity (Elmqvist et al. 2003; Tylianakis et al. 2010). Same

authors hypothesize that such an ecosystem would therefore be more resilient in the case of perturbation (Elmqvist et al. 2003; Tylianakis et al. 2010).

- the transitivity is the probability that two nodes are both directly and indirectly, i.e. using another node, connected (Girvan and Newman 2002). This metric

Fig. 3 Example of a bacterial co-occurrence network in a forest soil constructed from 16S RNA sequencing data (Karimi et al., unpublished data). The *points* correspond to nodes (connected *taxa*), the *green lines* indicate positive links (co-occurrence) and the *red lines* show negative links (co-exclusion). The size of the node indicates the relative abundance of the corresponding microbial taxa



indicates the level of clustering within the network. High transitivity is not incompatible with high connectivity. It indicates the presence of tripartite relationships within in the community, and more generally the existence of redundant pathways between nodes.

- the connectedness is the capacity of a network to link two nodes taken at random by at least one chain. This metric takes into account both the first degree relationships, i.e. without intermediate node and the indirect relationships, i.e. with at least one intermediate node. It is also an indicator of nodes clustering and network modularity. A higher connectedness implies more indirect pathways if the connectance is lower. From an ecological point of view, connectedness is an indicator of community cohesion. For example, a high network connectedness can be explained by a potential ecological niche shared by the organisms in a community.
- the last metric corresponds to the number of individual modules. These modules are observed if the connectedness is too weak and the network is divided in sub-parts.

Taxonomic co-occurrence networks as indicators of ecosystem biological quality

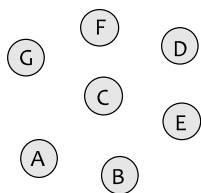
Taxonomic biodiversity, the usual indicator for environmental diagnosis

One of the indicators commonly used to assess the impact of natural or human-induced perturbations on the microbiological component of ecosystems is the measurement of taxonomic diversity (Bouchez et al. 2016). Estimates of taxonomic richness, Shannon diversity index and Simpson inverse, or evenness, are used to obtain standardized, synthetic measures of the effect of perturbations on the observed microbial diversity in terms of *taxa* presence and relative abundance (Hill 1973; Fierer and Lennon 2011). These indicators are of major interest due to the link between biodiversity and ecosystems functioning (Loreau et al. 2001). This link has been established for carbon cycle transformations such as the organic matter mineralization (Louis et al. 2016a; Baumann et al. 2012) or the production of methane (Ho et al. 2014), and also for key processes in the nitrogen cycle such as denitrification (Philippot et al.

Fig. 4 Schematic representation of the different metrics computed from the biotic interactions of co-occurrence networks architecture and the ecological meaning. Letters indicate microbial groups; red line indicates negative interaction; green line indicates positive interaction

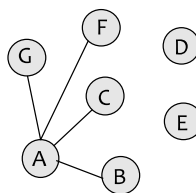
(a) Metrics at the scale of node

Relationship



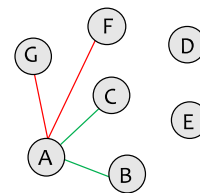
i- Connection
Is A connected or not to others units?

Complexity



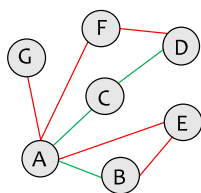
ii- Degree:
A is connected with 4 units. There are 4 links

Behavior



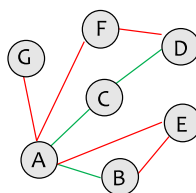
iii- Ratio positive/negative:
A is connected negatively with F and G and positively with B and C. The ratio for A is 1

Centrality



iv- Betweenness centrality:
A has the most direct and indirect connections. D and G are the least connected

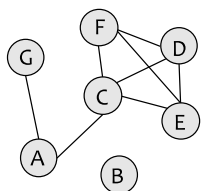
Key taxon



v- The maximal centrality:
A has the maximal centrality and is the key taxon.

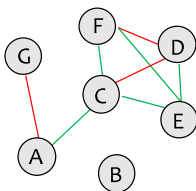
(b) Metrics at the scale of network

Potential relationships



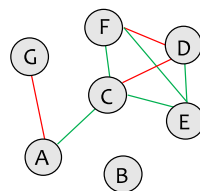
i- Number of nodes
7 units means 42 potential links. Only 6 are connected.

Quantity and quality of interactions



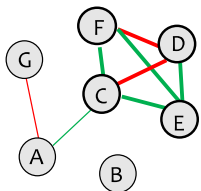
ii- Density and ratio:
8 links with a ratio of 5/3 in favor of positive links

Complexity



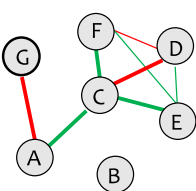
iii- Connectance:
Proportion of potential links which are realized (8/42)

Multiple pathways



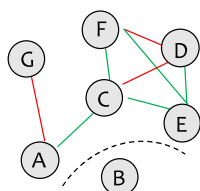
iv- Transitivity:
Proportion of tripartite relationships among the pathways : {C,D,E} {C,D,F} {D,E,F} {C,E,F}

Cohesion



v- Connectedness:
G and A are more distant from the other units and G is only indirectly connected to {C, D, E, F}.

Fragmentation



v- Number of modules:
2 modules {A,C,D,E,F} and {B}

2013) (Fig. 5). Thus, microbial diversity is now being integrated into new models to predict the transformations occurring in biogeochemical cycles (Louis et al. 2016b). Apart from its central role in nutrient cycles, microbial diversity can also regulate other functions such as plant diversity, carbon sequestration (Wagg et al. 2014), control of allochthonous populations' invasions (Vivant et al. 2013), mineralization of xenobiotic such as phenanthrene (Hernandez-Raquet et al. 2013) or the stability of ecosystem functioning in response to a perturbation (Tardy et al. 2014).

Despite its democratization in the field of ecology, the microbial diversity may not necessarily be informative or sensitive enough as an indicator of ecosystem state in response to perturbations (van Bruggen 2000; Karimi et al. 2016). Four non-exclusive hypotheses can be put forward to explain this:

- Firstly, although the microbial diversity is now known to be huge, most studies are still being focused on diversity that has already been identified and classified, and which represents the most cosmopolitan and dominant groups (Fig. 6a). These groups are therefore organisms that are able to adapt to a wide range of environmental conditions. Therefore, they may not be affected by perturbations while minor populations, in fact less readily detectable but potentially key in ecosystem functioning, are strongly modified. Thus, a change in diversity might not be perceptible with the tools currently available, even if these tools are becoming increasingly powerful.
- The second hypothesis concerns the intensity and occurrence rate of perturbations to ecosystems (Fig. 6b). Moderate and chronic disturbances correspond to real and current situations as well as to predictions for the future. The indicators of diversity do

not seem to be always sensitive to these realistic ranges of disturbance, however. Indeed, in the short term, a moderate perturbation tends to promote an adaptation and readjustment of the diversity within microbial communities (Tardy et al. 2015). If the diversity is measured after this period of readjustment, no change will be observed. Similarly, chronic perturbations can lead to stabilization of the diversity within a community. For example, despite one year of chronic atmospheric pollution in an urban or industrial environment, the taxonomic diversities of microbial communities associated with terrestrial bryophytes did not differ from those in an undisturbed rural environment (Karimi et al. 2016).

- The third hypothesis concerns the spatial scale used for the community and ecosystem observation (Fig. 6c). The taxonomic diversity measured will depend on the area sampled, in agreement with the “species–area” relationship (Ranjard et al. 2013), and tends to become more homogeneous at large scales (Cusson et al. 2014). For example, at a national scale, the impact of farming practices represents a weak proportion of the explanation of the variance in microbial diversity, but is much more meaningful at a local scale because the soil is less variable and therefore has less effect on the variation in biodiversity (Terrat et al. personal communication; Constancias et al. 2015).
- The last hypothesis concerns the indirect effects of perturbations on diversity (Wardle 2006). When a disturbance occurs, biotic interactions may be affected together with organisms and in turn affect the regulation of diversity (Vinebrooke et al. 2004) (Fig. 6d). One example of this regulation involves the prey of the mixotrophic amoeba *Hyalosphenia papilio*. If the number of prey (bacteria, ciliates, fungi, etc.) is reduced

Fig. 5 Schematic response of various ecosystem functions to microbial diversity level

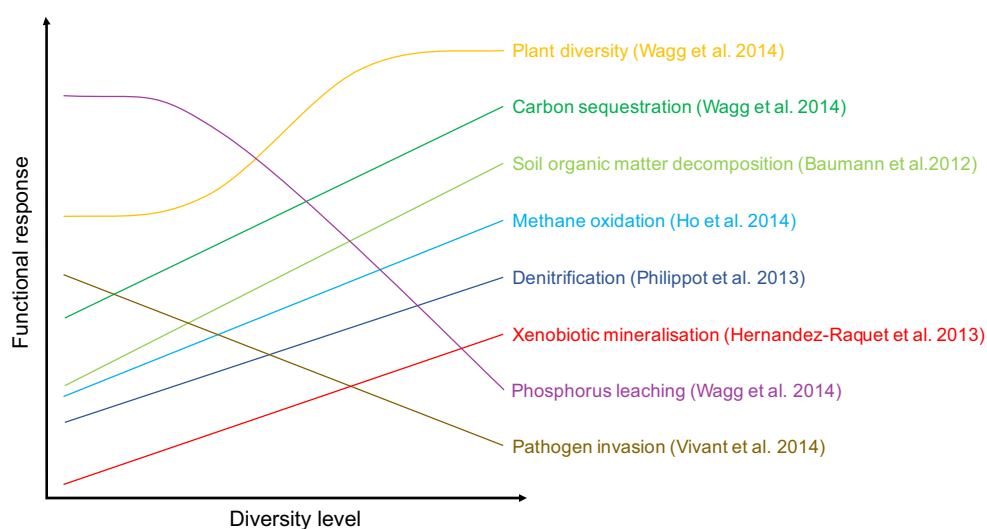
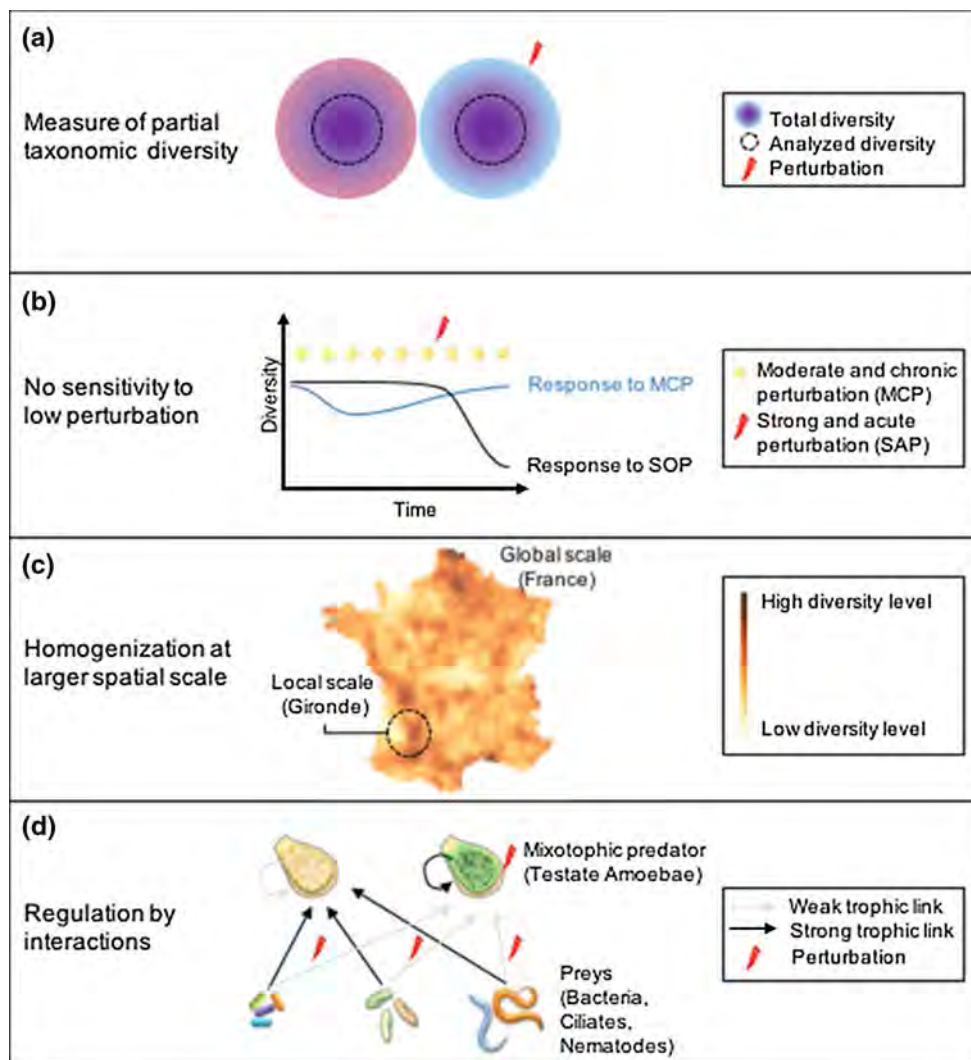


Fig. 6 Schematic representation of four hypotheses to decipher the lack of sensitivity of microbial biodiversity indicators in response to environmental perturbations



by an environmental perturbation, this predatory species uses its autotrophic ability and draws mainly its energy from photosynthesis. The reduction in the intensity of predation leads to a regulation of the microbial populations and potentially to a maintenance of diversity.

Microbial co-occurrence networks as indicator

To be relevant, a bioindicator of ecosystem state must (1) represent the intrinsic properties of the environment, (2) be interpretable beyond the information or measured parameter itself, (3) be derived from a standardized method, (4) be operational, i.e. be easy to set up, of moderate cost, and associated with a suitable reference system to allow interpretation and diagnosis, and finally, (5) be sensitive and specific to disturbances at scales of time and space relevant to human activities.

Sensitivity and specificity of co-occurrence networks

The co-occurrence networks approach and associated metrics have already been used in various studies to evaluate the effects of environmental disturbances. As indicated in Table 2, microbial co-occurrence networks are sensitive to atmospheric pollutions at the level of plant mats (Karimi et al. 2016), to metallic contaminants in the soil (Zappelini et al. 2015), to the type of land use (Lupatini et al. 2014; Zappelini et al. 2015) and more particularly to specific agricultural practices (Sauvadet et al. 2016). Apart from these examples, in situ experiments have shown that microbial networks in peatlands are sensitive, in the short term, to global warming (Karimi et al. Unpublished). A temperature increase of 1 °C for only 5 years led to a loss of complexity of the microbial co-occurrence network and a change in the central taxon from predatory amoeba to omnivorous ciliate. This switch is indicative of a potential modification in the trophic level of the ecosystem.

Table 2 Compilation of the recent papers demonstrating the sensitivity of co-occurrence networks to detect changes in microbial communities in response to perturbations in terrestrial ecosystems

Perturbation	Metrics	Network response
Atmospheric CO ₂ (Zhou et al. 2011)	Density of links	Ambient CO ₂ > Elevated CO ₂
	Transitivity	Ambient CO ₂ > Elevated CO ₂
Land use (Lupatini et al. 2014)	Density of links	Natural forest > Pasture > Field and plantation
	Connectedness	Natural forest < Pasture < Field and plantation
	Transitivity	No difference
Soil anthropization (Zappellini et al. 2015)	Density of links	Control > Contaminated tailings dump
	Connectance	Control > Contaminated tailings dump
	Average degree	Control > Contaminated tailings dump
	Average betweenness	Control > Contaminated tailings dump
	Connectedness	No difference
Litter quality (Sauvadet et al. 2016)	Density of links	Leaves(labile) > Roots(recalcitrant)
	Transitivity	Leaves(labile) > Roots(recalcitrant)
Atmospheric NO ₂ and PM ₁₀ (Karimi et al. 2016)	Density of links	Rural > Industrial > Urban
	Connectance	Rural > Industrial > Urban
	Ratio positive/negative	Rural > Industrial > Urban

Taken together, these studies validate the sensitivity of networks to a wide range of disturbances, including the chronic and moderate perturbations to which ecosystems are subjected. In situ studies carried out under complex multi-factorial environmental conditions have revealed the specificity of network responses to perturbation. In fact, Karimi et al. (2016) demonstrated that the structure of the microbial network in terrestrial bryophytes was sensitive—within a complex cocktail of chemical molecules—to atmospheric NO₂ and to the copper associated with particles larger than 10 μm in size. Although this suggests a definite specificity in the response of networks to disturbances, no clear empirical demonstration has yet been obtained.

From metrics of co-occurrence network to indicators

Co-occurrence networks provide a single holistic vision of ecosystems in that they integrate the direct and indirect effects of disturbances on the diversity, taxonomic composition and relationships between *taxa* within the community (Fig. 7). This approach, despite the complexity of the information that it synthesizes, relies on data that are now readily acquired by high-throughput sequencing. The mathematical implementation is relatively straightforward, well documented in the literature, and new methods are still being developed. Among these different methods, Berry and Widder (2014) recommend using the Spearman or Pearson correlation coefficient to identify the co-occurrence links within a community. However, the

standardization of a single method is impossible. In fact, the choice of method still depends on the quality of the biological data, the main aim being to reduce as much as possible the number of false-positives. Although the method used to construct co-occurrence networks can vary from one study to another, the metrics for quantifying network structure have been well defined (Figs. 4, 7). These metrics can therefore be used as synthetic indicators, easy to computed and interpretable from an ecological point of view. Each metric characterizes a different aspect of network structure and organization of the community but, as reported in the literature, not all of them are systematically sensitive to perturbations. Table 3 summarizes the network metrics that can be considered as relevant bioindicators of ecosystem state, according to the type of perturbation and the ecological interpretation of each bioindicator.

Zappellini et al. (2015) showed that anthropization of a soil, resulting from the addition of mercury-contaminated sediment, had a significant effect on four metrics (linkage density, connectance, average degree, average betweenness) without having an impact on the connectedness (Table 2). This response can be interpreted as a loss of complexity, a reduced level of community organization, but the maintenance of cohesion (same average lengths of the chains between *taxa*). In contrast, Lupatini et al. (2014) showed that the land use had an impact on connectedness and linkage density but not on network transitivity (Table 2). This signifies that the network was less complex, with weaker cohesion (the chains between *taxa* pass by

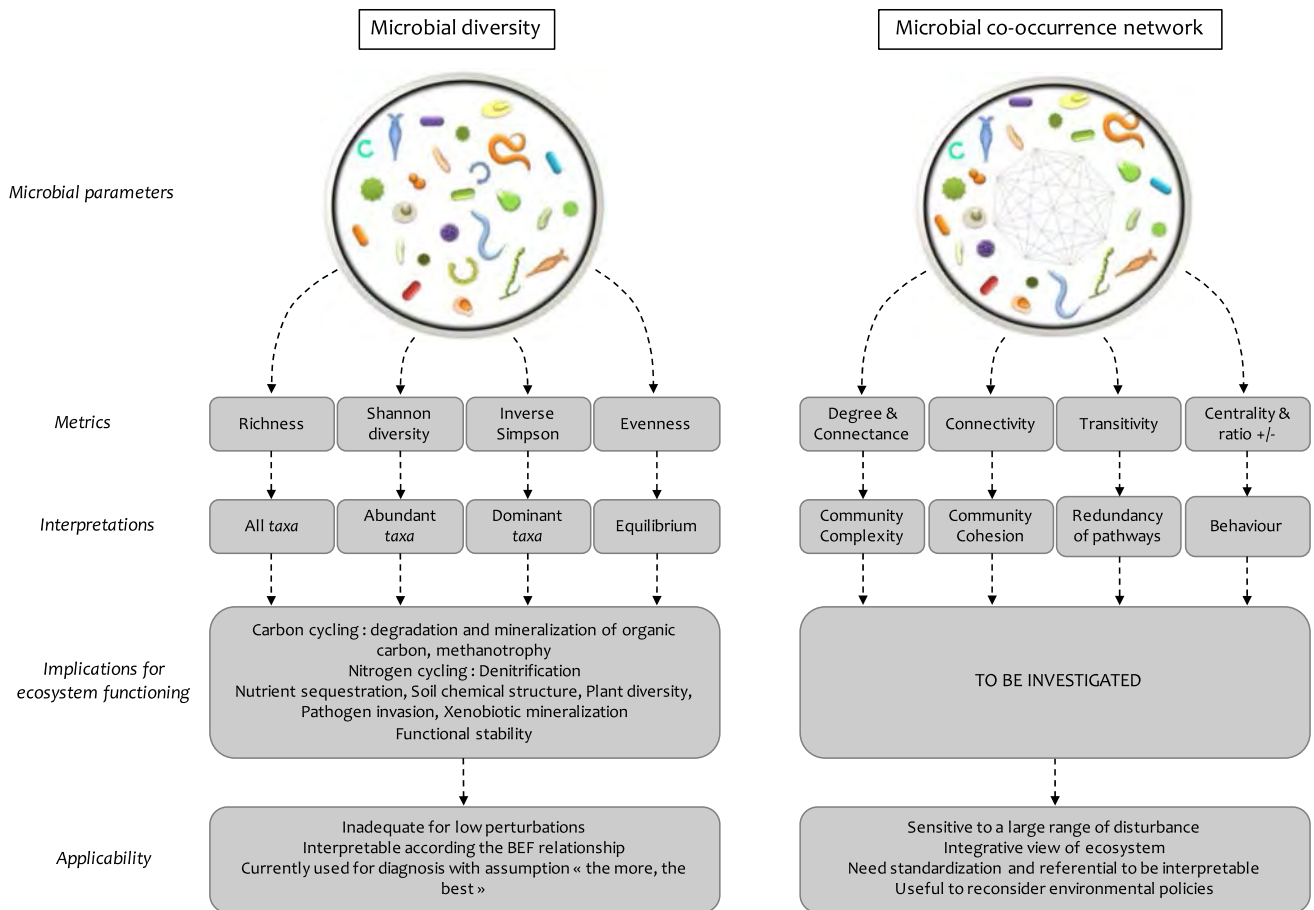


Fig. 7 Schematic representation of strengths and limits of microbial diversity and co-occurrence network as indicators of environmental quality. *BEF* biodiversity–ecosystem functioning

Table 3 Potentiality of the co-occurrence network metrics to be used as indicators of microbial response to perturbations

Metrics	Ecological meaning	Bioindication of...				
		Global warming ^a	Atmospheric pollution ^{b,c}	Soil anthropization ^d	Land use ^{d,e}	Agricultural practices ^{a,f}
Linkage density	Diversity of relationship in the community	x	x	x	x	x
Ratio positive/negative	Cooperation level within the community	x	x	x	x	x
Connectance	Organization level of community	x	x	x	x	x
Connectedness	Indirect relationship and level of cohesion of the community	x		x	x	x
Transitivity	Amount of threesome <i>taxa</i> and multiple pathways relationships	x	x			x
Average degree	Complexity of the network	x	x	x	x	x
Maximal betweenness	Keystone <i>taxon</i>	x	x	x	x	

^a Karimi et al. (unpublished data), ^b Zhou et al. (2011), ^c Karimi et al. (2016), ^d Zappelini et al. (2015), ^e Lupatini et al. (2014), ^f Sauvadet et al. (2016)

more intermediates) but exhibited the same level of clustering of the *taxa* into triplets. A major result in these studies was that when an environment was disturbed the

complexity of the microbial co-occurrence network was reduced (Table 2), as indicated by the reduction in linkage density, connectance and average degree. These three

metrics might therefore be suitable indicators of ecosystem state. Until now, the only metric that has been systematically calculated and presented as an indicator of state is linkage density and the other metrics are used less often. In parallel, the connectedness and transitivity responses, which provide information about the cohesion and clustering into small groups, are dependent on the type of perturbation. Thus, these metrics are also necessary as indicators of state to measure the complete and complex response of the network to disturbance. Although some metrics were more usually computed, it would be more cautious to use the largest set of indicators to better assess the response of microbial networks to disturbance.

The studies presented here provide the first reference values for using networks as tools for environmental diagnosis. Altogether, they suggest, in an empirical manner, that microbial networks of greater complexity are characteristic of the least disturbed ecosystems. Nevertheless, for these networks to be efficient and operational indicators of ecosystem state, appropriate reference systems taking into account the type and intensity of the perturbations and the spatial and time scales of investigation will also need to be established for each of the environmental matrix investigated.

Microbial network as indicator of biological functioning?

The relationship between biotic interactions networks and ecosystem functioning is most clearly apparent for interactions centred on resources, such as trophic or mutualistic networks (Brussaard et al. 2004), but also exists for other functions such as the protection of roots by a complex system of microbial interactions (Jousset et al. 2009), or the development of specialized biofilms (Hansen et al. 2007). Historically, the link between ecological networks and biological functioning of the ecosystem has been studied for macro-organisms. The interactions network approaches applied to these organisms demonstrated that the resilience of ecosystems and the stability of their functions are directly linked to taxonomic diversity and to interactions between the species (Schmid et al. 2009). Theoretical studies showed that in the case of disturbance, interactions are the first to be affected and thus alter the functions of the ecosystem even before the species disappear (Valiente-Banuet et al. 2015). Certain authors even proposed that a modification in network structure could lead to an alteration in the functioning of ecosystems and to a reduction in their stability (resistance/resilience) in the long term (Tylianakis et al. 2010; Vacher et al. 2016).

Therefore, the analysis of co-occurrence networks in microbial communities could also be used as an early indicator of the functional response of an ecosystem. In

terms of ecosystem stability, the complexity of the network might be linked, as indicated above for macro-organisms, to the resistance and resilience of the community to perturbations. The structure of the microbial network could be an indicator of the functions of decomposition, denitrification or linked to the production of plant biomass, just like taxonomic diversity but with greater reactivity and sensitivity. It has already been shown that a less complex microbial network releases fewer CO₂ emissions (Blouin et al. 2015), which suggests a greater storage of carbon, but this is the only study to link network with microbial function.

Conclusion

Despite all the questions that remain unanswered, microbial networks and particularly the co-occurrence networks of microbial *taxa* provide promising tools for evaluating the state and stability of ecosystems. Even if the link has not yet been established between the characteristics of microbial networks and specific ecosystem functions, networks might also be relevant bioindicators of functioning. As the classically used indicators of diversity present certain limitations, co-occurrence networks could provide complementary information about ecosystems and thus serve as early, more sensitive bioindicators (Bouchez et al. 2016).

If future studies validate the essential points associated with specificity, reference systems and link with function, they should make it possible to identify impacts that have been imperceptible until now, and possibly challenge some practices authorized by current environmental policies (Karimi et al. 2016). This ecosystem “imprint” represents a promising workable and readily mobilized new generation bioindicators for evaluating the quality of ecosystems in environmental diagnoses.

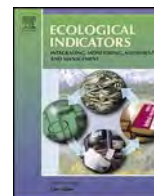
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Predictive model of soil molecular microbial biomass



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ABSTRACT

Preservation and sustainable use of soil biological communities represent major challenges in the current agroecological context. However, to identify the agricultural practices/systems that match with these challenges, innovative tools have to be developed to establish a diagnosis of the biological status of the soil. Here, we have developed a statistical polynomial model to predict the molecular biomass of the soil microbial community according to the soil physicochemical properties. For this, we used a dataset of soil molecular microbial biomass estimates and pedoclimatic properties derived from analyses of samples collected in the context of the “French monitoring soil quality network = Réseau de Mesures de la qualité des Sols” (RMQS). This sampling network has provided 21 15 soil samples covering the range of variability of soil type and land use at the scale of France. The best model obtained from the data showed that soil organic carbon content, clay content, altitude, and pH were the best explanatory variables of soil microbial biomass while other variables such as longitude, latitude and annual temperature were negligible. Based on these variables, the multilinear model developed allowed very accurate prediction of the soil microbial biomass, with an excellent adjusted coefficient of determination R^2_{adj} of 0.6772 ($P < 10^{-3}$). In addition to R^2_{adj} , the model was further validated by results from cross validation and sensitivity analyses. The model provides a reference value for microbial biomass for a given pedoclimatic condition, which can then be compared with the corresponding measured data to provide for the first time a robust diagnosis of soil quality. Application of the model to a set of soil samples obtained at the scale of an agricultural landscape is presented and discussed, showing the suitability of the model to diagnose of the impact of particular agricultural practices such as tillage and catch crops in field conditions, at least over the French nation.

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1. Introduction

Soil is the support for human constructions and agricultural production. At the interface with other compartments of the biosphere, soil fulfills numerous functions essential for the provision of many ecosystem goods and services necessary to the well being of human societies (Millennium Ecosystem Assessment, 2005). It is also a non-renewable resource, whose physicochemical and biological properties have been altered by overexploitation for the development of intensive agriculture and industrialization (Giller

et al., 1997; Thiele-Bruhn et al., 2012). The increasing recognition of this situation has revealed a need to define new modes of land management, which are adapted to the preservation and sustainable use of soils (Rames et al., 2013). To attain this objective, however, the ability to evaluate the effects of agricultural practices on soil “biological quality” needs to be improved and the developing of a suitable set of indicators would represent a decisive step forwards (Rames et al., 2013; Pulleman et al., 2012; Ritz et al., 2009).

Most of the ecosystem services provided by soil results from biological and ecological processes (i.e. nutrient cycling, soil aggregation, depollution, etc.) driven by taxonomic and functional assemblages of the indigenous biological communities (Coleman and Whitman, 2005). Consequently, soil biological properties are logical candidates as effective indicators of soil quality and

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sustainability (Ritz et al., 2009). To help establish a robust diagnosis of soil quality, biological indicators have to be sensitive to management changes and relevant to soil functions such as nutrient cycling (Rames et al., 2013; Pulleman et al., 2012). In this regard, soil microbial communities offer particularly great potential since (i) they provide a major contribution to organic matter decomposition and nutrient transformation, acting as soil “chemical engineers”, and (ii) they respond with great sensitivity to environmental and management-induced changes through modification of their biomass, structure/diversity, and activity (Pulleman et al., 2012; Sharma et al., 2011). However, good indicators also need to satisfy technical, practical and economic prerequisites (*i.e.* to be simple, rapid, reproducible, cheap, high-throughput, etc.), and to be associated with references. These references constitute an operating range (low/normal/high) in which measured values are positioned in order to perform the desired diagnosis (Pulleman et al., 2012; Ritz et al., 2009). Although many of the biological methods developed over the past twenty years for soil microbial communities characterization (Maron et al., 2011) have been proposed as potential indicators of soil quality, very few meet all these criteria (Ritz et al., 2009). Most patently apparent is the lack of (i) standardized procedures, and (ii) references associated with these indicators.

In this context, determination of the microbial biomass by quantifying the DNA extracted from soil is certainly one of the most promising of all the microbial indicators available. Microbial biomass has long been recognized as a suitable indicator of soil quality (Garcia and Hernandez, 1997; Horwath and Paul, 1994; Harden et al., 1993). However, the procedure historically used for its measurement (*i.e.* the fumigation-extraction method; Vance et al., 1987), although standardized, was time consuming and laborious, and made difficult to establish of a reference system. Recently, direct extraction and quantification of DNA from soil has been shown to be a robust, fast and easy way of estimating the size of the soil microbial pool (Fornasier et al., 2014; Gangneux et al., 2011; Terrat et al., 2012; Dequiedt et al., 2011; Ascher et al., 2009; Marstorp and Witter, 1999). One noteworthy advantage of this method is that it is rapid and can be deployed at high throughput. It was therefore used to estimate the microbial biomass in 2115 soil samples from the French Soil Quality Monitoring Network (‘Réseau de Mesures de la Qualité des Sols’, RMQS, Dequiedt et al., 2011), which covers the full range of variability of soil type and land use at the scale of the French national territory (Arrouays et al., 2002). From an ecological point of view, these latter studies provided new insights into the spatial distribution of microbial abundance, as well as of the drivers (soil physicochemical properties/climatic factors/land use) explaining the observed patterns. It also led to establishment of a highly representative dataset of microbial biomass at wide spatial scale.

Based on this reference, our aim in this study was to develop a statistical predictive model of soil microbial biomass according to environmental parameters including soil physico-chemical and climatic characteristics. This model is an innovative tool providing a reference value of microbial biomass for a given pedoclimatic condition, which can then be compared with the corresponding measured data to allow a robust diagnosis of soil quality. In the context of environmental evaluation of land management, the association of this diagnosis with a reference range of variation, should provide the validation of molecular microbial biomass as a robust and operational bioindicator of soil biological quality. By applying our model to soil information for a given agricultural landscape we also demonstrated its ability to estimate the impact of particular agricultural practices such as tillage and catch crop in real soil management conditions and environmental heterogeneity.

2. Materials and methods

2.1. Soil and environmental dataset

The sampling network used in this study to obtain numerous, representative and spatially distributed soil and environmental data at the scale of France was the French Soil Quality Monitoring Network called RMQS (“Réseau de Mesures de la qualité des Sols”). This network is based on a $16\text{ km} \times 16\text{ km}$ systematic grid covering the whole of France (Arrouays et al., 2002) and includes 2115 sites, each located at the center of a $16\text{ km} \times 16\text{ km}$ cell. The sample of all the soils at the national scale was carried out from 2002 to 2009. For each year, samples were collected from March to September. During this period, samples were not collected when soils were exposed to extreme climatic conditions (*i.e.* drought during summer) to avoid possible biases. All sites have been geo-positioned with a precision $<0.5\text{ m}$ and the soil profile, site environment, climatic factors and land-use described. In the middle of each $16\text{ km} \times 16\text{ km}$ cell, 25 individual core samples (7 cm in diameter) were taken from the topsoil (0–30 cm) using an unaligned sampling design within a $20\text{ m} \times 20\text{ m}$ area. The core samples were bulked to obtain a composite sample for each site. The soil samples were air-dried (controlled conditions, constant temperature 30°C), sieved to 2 mm and stored at -40°C before analysis. Several physico-chemical parameters were measured on each soil *i.e.*, particle-size distribution (3 classes: sand: 2000–50 μm , silt: 50–2 μm , clay: $<2\text{ }\mu\text{m}$, NF X 31-107), pH water (NF ISO 10390), C_{org} (dry combustion NF ISO 10694), N (dry combustion NF ISO 13878), C:N ratio, soluble P contents (Olsen method NF ISO 11263), CaCO_3 (volumetric method NF ISO 10693), CEC (extraction with cobaltihexamine chloride, NF X 31-130) and exchangeable cations (Ca, Mg, extraction $\text{HF} + \text{HClO}_4$, NF ISO 14689-1). Physical and chemical analyses are available for all 2115 samples and were performed by the Soil Analysis Laboratory of INRA (Arras, France, <http://www.lille.inra.fr/las>). The climatic data were annual rainfall (Rain_year), annual evapotranspiration (ETP_year) and annual temperature (Temp_year), and were obtained by a spatial intersection between the RMQS grid and the 8 km grid produced by the SAFRAN model (Quintana-Segui et al., 2008). Land use was recorded according to the CORINE Land Cover classification (IFEN, <http://www.statistiques.developpement-durable.gouv.fr/>) and with 7 classes: forest, crop systems, grasslands, particular natural ecosystems, vineyards/orchards, parkland and wild land.

2.2. Molecular microbial biomass dataset

The soil molecular microbial biomass of each RMQS sample was determined using the Gns-GII procedure optimized by the GenoSol platform (http://www2.dijon.inra.fr/plateforme_genosol, Plassart et al., 2012; Terrat et al., 2015). Crude DNA extracts were resolved by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide and photographed (Biocapt, Vilber Lourmat, Marne la vallée, France). Dilutions of calf thymus DNA (BIORAD) were included in each gel and a standard curve of DNA concentration (1000, 500, 250, 125, 62.5 to 31.25 ng) was used to estimate the final DNA concentration in the crude extracts (Ranjard et al., 2003). The ethidium bromide intensity was integrated with ImageQuaNT software (Molecular Dynamics, Evry, France). The reliability of this method to limit bias due to soil impurities that can hamper DNA quantification has been confirmed (Ranjard et al., 2003).

2.3. Mapping of soil DNA recovery

A map of DNA recovery was produced by applying the same method of geostatistical interpolation used in the previous work of Dequiedt et al. (2011). After an appropriate transformation of

the data, a robust estimation of the variogram was performed and fitted by a Matérn function. Finally, the map of DNA recovery was produced after backtransforming the predicted median by ordinary kriging. The validity of the fitted geostatistical model was assessed in terms of the standardized squared prediction errors using the results of a leave-one-out cross-validation.

2.4. Modeling strategy

Polynomial regression analyses were carried out to model soil molecular microbial biomass (response variable) as a function of soil physico-chemical characteristics, geographical coordinates and climatic data (explanatory variables). In this approach, pH was transformed into $[H_3O^+]$. In order to find the best explanatory and most parsimonious model, the modeling steps were: first to identify the collinear explanatory variables to be excluded from the analysis; second to select the best set of explanatory variables and the best model form based on predictive capabilities through R^2_{adj} and cross-validation; and third to assess model robustness regarding measurement errors on explanatory variables. Finally, the sensitivity of the predicted microbial biomass to the model's parameters was evaluated and the errors associated with the errors on explanatory variables were quantified. The whole modeling strategy was developed in R software (R Development Core Team, 2011).

Two tools were used to assess collinearity between the explanatory variables, namely correlation coefficients and variance inflation factors (VIF). Only explanatory variables with a correlation coefficient ranging from -0.7 to 0.7 and with a $VIF \leq 4$ were considered in the modeling steps. The VIF values were calculated using the `vif` function in the `car` package (Fox, 2008). Since the number of explanatory variables was not very large (<50), the best explanatory variables was selected according to the exhaustive search method described by Miller (2002). This approach involved using the `regsubsets` function in the `leaps` package in R (Moore, 1995). The selection criteria were both the Bayesian Information Criterion (BIC) and the adjusted coefficient of determination (R^2_{adj}), and involved minimizing the first and maximizing the second. Two additional selection criteria were considered: the Akaike Information Criterion (AIC) and Mallows's C_p , which lead to the same conclusions (data not shown).

Based on the selected set of explanatory variables, the molecular microbial biomass dataset was randomly divided into a training dataset (90% of the data, 1879 soil samples) and a cross-validation dataset (about 10% of the data, 236 soil samples) using the KennardStone algorithm in the `kenStone` in `prospectr` package (Stevens and Ramirez-Lopez, 2013). The distribution of training and cross-validation datasets is shown in Fig. S1. Different models of increasing complexity were then compared, ranging from simple linear models to polynomial forms of increasing degree that included interactions between explanatory variables. The polynomial regression form was chosen because it had the advantage of being easy to implement and flexible. Nevertheless, the polynomial degree had to be determined carefully to avoid model over-learning since adding higher order terms in a model improves the fit to the data but provided poorer predictions on new datasets. Model selection was therefore based on maximizing R^2_{adj} , while minimizing BIC and by cross-validating the model on the cross-validation dataset. This approach was applied by using two algorithms specifically developed for this study and implemented in R. The first one calculates the predicted soil molecular microbial biomass given the selected explanatory variables. The second one manages data input, executes the first algorithm, and retrieves predicted values directly from Excel® software.

2.5. Sensitivity analysis of the model

The sensitivity analysis was used to evaluate the range of variation of model predictions according to little measurement errors on the descriptors. The sensitivity of the model was estimated by means of a sensitivity index. Since the basis of the model was the linear regression, standardized regression coefficients (SRC) were used as sensitivity indexes, as classically observed in the literature (Saltelli et al., 2008). The regression coefficients denoted by $\hat{\beta}$ were determined by ordinary least-squares regression and provide information about the sensitivity of the model response to the various input-factors, and their combinations. SRC is equal to $(\sigma_{X_i}/\sigma_Y)\hat{\beta}$, where σ_{X_i} , σ_Y are the standard deviation of inputs and output variables; respectively. The SRC values were determined using the `sensitivity` package in R (Saltelli et al., 2000). With this approach, the sensitivity of the model to a given variable is high when the absolute value of SRC is high.

2.6. Validation of the model with an independent dataset

The model developed was used to predict microbial biomass in 264 soils sampled across an agricultural landscape for which the soil and land use characteristics had been accurately determined and the molecular microbial biomass estimated with the same techniques as for the RMQS samples (Constancias et al., 2015a). The area 13 km^2 wide is located in Burgundy (Fénay, Lat: $47^\circ 14' 37'' \text{ N}$, Long: $5^\circ 03' 36'' \text{ E}$, Burgundy, France) and characterized by a smaller variability in soil properties compared to RMQS, and also by a mosaic of different types of land-use constituted by oak-hornbeam deciduous forests (3.86 km^2) and agricultural croplands with contrasting cropping intensity and especially soil tillage (9.22 km^2), cultivated essentially with winter crops (winter wheat, oilseed rape) in rotation with late-sown crops (spring barley). The site is under continental climate, with a mean annual air temperature of 10.4° C and a mean annual rainfall of 762 mm (period 1968–2011). The sampling design covers the entire area and is based upon a square grid with a spacing of 215 m , which corresponds to 264 sites. All sites were sampled in September 2011. At each of the 264 sampling locations, five soil cores (core diameter: 5 cm ; $0\text{--}20 \text{ cm}$ depth) were randomly collected over an area of 4 m^2 at inter-row for agricultural sites and at least 1 m away from trees in deciduous forests, then bulked, 2 mm -sieved before being lyophilized at -80° C and finally stored at -40° C . Analyses of physicochemical properties (pH, organic carbon, total nitrogen, CaCO_3 , clay, silt and sand) were performed by the Laboratoire d'analyse des sols d'Arras of INRA (www.lille.inra.fr/las) as described in Dequiedt et al. (2011). Land management practices over the entire landscape were summarized by means of a factorial analysis for mixed data (Constancias et al., 2015a). This analysis was performed using the `FactoMineR` package (Lê et al., 2008) and allowed the definition of land management clusters. The input data were land use, soil tillage, crop rotation diversity (number of plant types in the crop rotation) and pesticide treatment frequency index. Six clusters were finally defined, mainly based on soil tillage intensity and inter-cropping: conventional tillage, mechanical hoeing, minimal tillage, catch crops, perennial crops, and forests.

3. Results and discussion

3.1. Variation of molecular microbial biomass on the scale of France

The method of DNA extraction initially used to recover soil DNA in large-scale RMQS soil surveys (Ranjard et al., 2009; Dequiedt et al., 2011; Fig. S2), has recently been further improved to increase

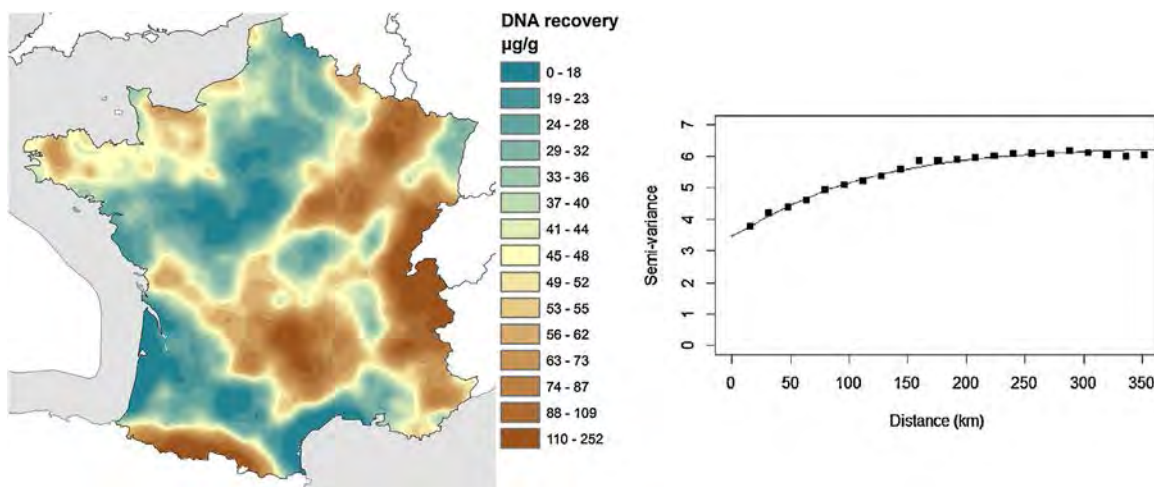


Fig. 1. Mapping of soil molecular microbial biomass at the scale of the French territory. Soil molecular microbial biomass was determined directly from the quantification of soil DNA and was interpolated through a standard geostatistical approach.

the yield of DNA extraction (Terrat et al., 2012) together with the representativeness of the extract in terms of taxonomic diversity of soil microbial communities (Terrat et al., 2012, 2015). This new procedure (GnS-GII) was used to quantify newly the microbial biomass from 2115 RMQS soil samples, and provided an updated dataset of microbial biomass.

Although, the soil DNA yield obtained with GnS-GII was greater than that reported by Dequiedt et al. (2011), the distribution pattern observed at the scale of France was very similar (Fig. 1). The map obtained from a geostatistical interpolation of the recovered DNA confirmed the heterogeneous distribution of microbial biomass at the scale of France reported in Dequiedt et al. (2011). In addition, the fitted parameters of the Matérn model yielded good cross validation (median of standardized squared prediction errors=0.455), thereby confirming that molecular microbial biomass is spatially organized in biogeographical patterns covering about 100 km, similar to those reported previously by Dequiedt

et al. (2011). The size of these biogeographical patches confirmed that variation of soil types based on their physico-chemical characteristics rather than changes of global climatic factors may have a strong influence on the distribution of the microbial biomass at the scale of France (Dequiedt et al., 2011). This finding justified our strategy to develop a statistical predictive model of microbial biomass based essentially on soil pedo-climatic conditions to better evaluate the impact of land use on soil microbial abundance. In other respects, our results evidenced that this new procedure did not bias the difference in microbial biomass between soils. However, it proportionally increased the amount of DNA extracted from each soil (about 6 times higher with a range of variation from 0.05 to 107 times). This difference of yield between the two DNA extraction procedures is explained by a better lysis of fungal populations leading to higher recovery of fungal DNA (Terrat et al., 2015). As a consequence, the range of variation may be also explained by the various fungal relative abundance between the RMQS soils. The

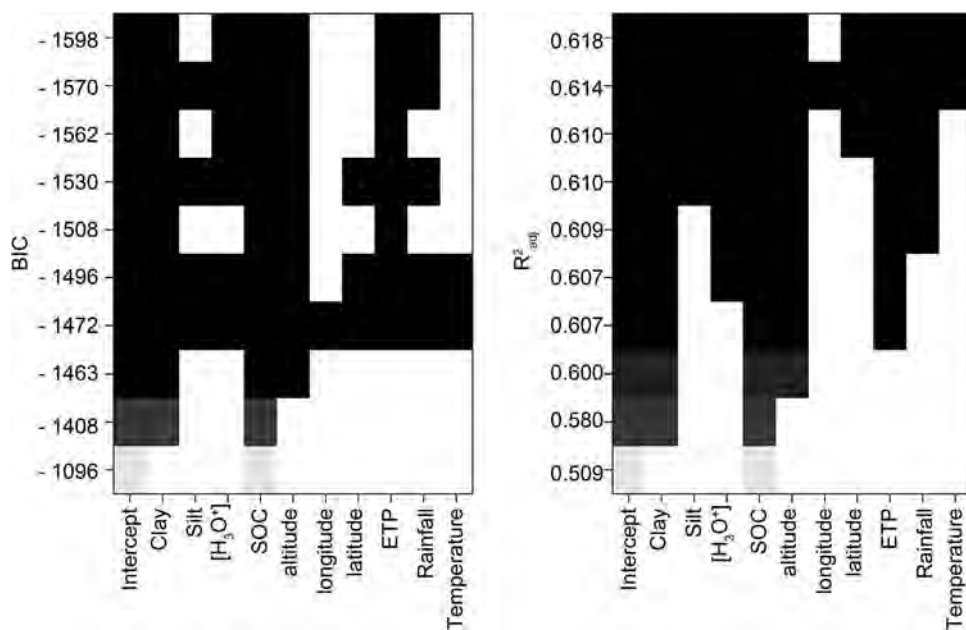


Fig. 2. Hierarchy of the linear models of soil microbial biomass involving soil physicochemical, spatial and climatic variables according to the BIC and the R^2_{adj} with the exhaustive method. (A) BIC criterion, (B) R^2_{adj} criterion. Each row in this graph represents a specific model. The variables included in a given model are represented by means of shaded rectangles. The intensity of the shading represents the ordering of the BIC and R^2_{adj} values according to the absolute value.

new amounts of recovered DNA ranged from 0.1 $\mu\text{g DNA g}^{-1}$ soil to about 630 $\mu\text{g DNA g}^{-1}$ soil with a mean of 42.4 $\mu\text{g DNA g}^{-1}$ soil.

3.2. Selection of environmental descriptors as explanatory variables of the predictive model

The environmental descriptors that most significantly influence soil microbial biomass were identified and ranked by applying the BIC and R_{adj}^2 methods. The multilinear model with the lowest BIC (−1409) and the highest R_{adj}^2 (0.587) involved six variables that were significantly good predictors of the molecular microbial biomass (Fig. 2). More precisely, the influence of the environmental descriptors could be ranked as follows according to the standardized regression coefficients: soil organic carbon content (SOC) = 0.57 > clay = 0.42 > altitude = 0.23 > annual evapotranspiration (ETP_{annual}) = 0.21 > pH 0.18 > annual rainfall = 0.14. To elaborate a predictive model of microbial biomass, we selected four explanatory variables without retaining any climate data in spite of their important role (particularly that of evapotranspiration). This choice was motivated by our desire to keep the model as much operational as possible. Indeed, climatic descriptors are not easily available and are expensive, which may have precluded the use of this model in diagnoses for and by soil users. In addition, the robustness of the model was not significantly altered ($P < 10^{-4}$) by substituting the evapotranspiration data with pH data (R_{adj}^2 of 0.539 with ETP and of 0.509 with pH) or therefore the quality of the prediction.

Interestingly, the best environmental descriptors identified in this study were similar to those identified in Dequiedt et al. (2011) and in several other studies (Bååth and Anderson, 2003; Mulder et al., 2005; Johnson et al., 2003) at a continental scale. In a recent study, Serna-Chavez et al. (2013) also identified soil moisture as a major driver of soil microbial biomass. We did not directly measure moisture here, but our results may be in agreement with these authors since SOC and clay, two of the main drivers identified in our study, are also usually highly correlated with soil moisture. In addition, it is important to emphasize that soil altitude, considered as an important driver in our model, is integrative of environmental parameters, especially climate and soil moisture conditions at the scale of France (Arrouays et al., 2002).

3.3. From multiple to polynomial linear models to predict molecular microbial biomass

In a first step, we tested the accuracy of the multiple linear model without considering interactions between the four explanatory variables SOC, clay, altitude and $[\text{H}_3\text{O}^+]$ previously identified and ranked (Fig. 2) on the 1879 samples of the modeling dataset. The adjusted coefficient of determination R_{adj}^2 was 0.5377 ($P < 10^{-4}$), which was considered sufficient to obtain a good prediction of molecular microbial biomass (Table S1 in supplemental information). However, the results also showed a non-normality of the model residuals (Fig. S3A in SI), which was confirmed by a significant Shapiro–Wilk test of normality ($P < 10^{-4}$). Considerable heterogeneity of the residuals was also observed (Fig. S3B in SI). Since the residuals were not normally distributed with a mean of 0 and constant variation, the multiple linear model could not be validated (Cornillon and Martzner-Løber, 2011).

In a second step, a polynomial regression model was chosen since this was expected to increase R_{adj}^2 to get closer to the normality hypotheses and to improve the homogeneity of the residuals as compared to the multiple linear model. In addition, increasing the model complexity was also tested for its ability to include interactions between the explanatory variables (Storlie and Helton, 2008). To identify the most valuable type of model, predicted values of

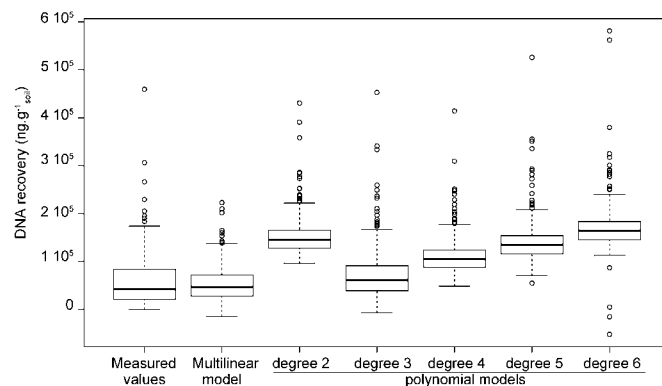


Fig. 3. Boxplot representation of the microbial biomass measured and predicted by a multiple and by polynomial linear models of increasing complexity degree. For each boxplot, the bold line represents the median of the values, the sides of the box represent the first and third quartiles and the error bars represent the standard deviation of the mean. Open circles correspond to outlier values in the normal distribution. The first two boxplots refer to the measured soil microbial biomass and to the soil microbial biomass predicted by means of a multiple linear model. The five boxplots on the right represent the distribution of the predicted soil microbial biomass by means of a polynomial model of increasing degree (from 2 to 6; respectively).

microbial biomass obtained by models of increasing complexity (from a multiple linear model to a sixth degree polynomial model) were confronted with measured values using the cross validation dataset (this latter being represented by the 10% of the RMQS dataset, Fig. 3). Results showed that the multiple linear model allowed correct prediction of the overall average soil biomass content but failed to attain the high values and tended to increase the number of negative predicted values compared to the other polynomial models. Increasing model complexity did not initially improve the quality of the prediction since the second degree polynomial lead to a strong over-estimation of microbial biomass (Fig. 3). This can be explained by the smaller number of parameters included in this polynomial model compared to the other polynomial degree together with the fact that all these parameters were positive. A further increase of model complexity with a third degree polynomial model substantially improved the quality of the prediction. The overall average soil molecular microbial biomass and the range of biomass variation from high to low (but not negative) values was well reproduced (Fig. 3). Better results were obtained with the third degree polynomial model than with the second degree polynomial model. This can be explained by the more number of parameters taken into account together with the fact that some of these parameters were negative, hence precluding over-estimation. However the results clearly evidenced that a further increase in the degree of the polynomial model was inappropriate since it lead to a decrease in prediction quality (Rawling et al., 1998), mainly characterized by an increased of the over-estimation of the soil microbial biomass (Fig. 3). In addition, due to the general properties of polynomial models, the coefficient estimates in polynomial models with a degree higher than 3 were not robust due to the increased sensitivity of the prediction to the removal of one or more data.

In the light of these results, we selected the third degree polynomial model since it gave the best prediction of soil microbial biomass. This model has the following mathematical form (Eq. (1))

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^4 \beta_{iii} X_i^3 + \sum_{i=1}^2 \sum_{j=i+1}^3 \sum_{k=i+1}^4 \beta_{ijk} X_i^j X_j^k + \sum_{i=1}^3 \sum_{j=i+1}^4 \beta_{ij} X_i X_j^2 + \sum_{i=1}^3 \sum_{j=i+1}^4 \sum_{k=i+j}^4 \beta_{ijj} X_i X_j X_k + \varepsilon \quad (1)$$

where Y is the estimated response variable (soil molecular microbial biomass) for the selected set of input factors or explanatory variables X_i ($i \in \{1; 2; 3; 4\}$), which are SOC, clay, altitude, and $[\text{H}_3\text{O}^+]$. X_i^2 and X_i^3 represent the quadratic and the cubic variables, respectively. $X_i X_j$ and $X_i X_j X_k$ are the multiplicative interaction terms. α_0 is the overall mean. By applying the polynomial model to the modeling dataset of 1879 samples, we obtained an excellent adjusted coefficient of determination R_{adj}^2 of 0.6738 ($P < 10^{-5}$) and minimal BIC (-1788), which was much higher than the R_{adj}^2 of 0.5847 ($P < 10^{-5}$) and lower than the BIC of -1598 obtained with the multiple linear model, respectively. In addition, we evidenced a normality of the residuals distribution, which was confirmed by the P -value of the Shapiro–Wilk test of normality ($P = 0.144 > 0.05$, Fig. S4B), as well as good homogeneity of the residuals (Fig. S4A). Based on these criteria, the third degree polynomial model was validated. Table 1 presents all the terms in the model with their corresponding coefficients, standard error and significance. Many terms in the model did not have a significant effect, but were conserved since the reference RMQS database is a monitoring network that can evolve by the addition of other sites and environmental variability, which could potentially make some of these variables significant.

3.4. Analysis of model sensitivity

In the context of developing a new predictive model it was crucial to evaluate its sensitivity to explanatory variables and to estimate the error on the estimated microbial biomass resulting from measurement errors on each explanatory variable. The sensitivity analysis enabled a sensitivity index to be retrieved for each

Table 2

Overview of the model sensitivity analysis. The standardized regression coefficients (SRC) of the variables to which the model is most sensitive are presented here. A complete sensitivity analysis is provided in Table S1. The variables are ordinated according to the absolute value of their associated SRC from the highest to the lowest.

Parameters	SRC
Interaction (clay:pH)	2.18
Interaction (clay:carbon)	1.79
Interaction (carbon:pH)	1.23
Interaction (clay:pH ²)	-1.15
pH (quadratic effect)	1.05
Carbon (quadratic effect)	1.03
Carbon	-0.95
Interaction (carbon:pH ²)	-0.84
Clay	-0.74
pH	-0.64

component of the model (overview of the most important components in Table 2 and complete results in Table S1). This analysis demonstrated that the model was highly sensitive to variations of clay content, carbon content and pH, together with their interactions and quadratic effects. This was in agreement with the literature since variations in clay content, *i.e.* following a gradient from coarse to fine textured soils, are supposed to modulate the size of microbial habitats (Dequiedt et al., 2011; Constancias et al., 2015a) and since variations in soil Carbon content provide an overview of resource availability for soil microbial growth (Serna-Chavez et al., 2013; Constancias et al., 2015a,b) while soil pH determines the level of enzymatic activities involved in retrieving growth substrates from the environment (Lauber et al., 2009).

The sensitivity analysis also suggests that robust measures of soil pH, soil carbon and clay content are required to obtain robust

Table 1

Summary of model coefficients and significance. This table describes the coefficients $\hat{\beta}$ of the third degree polynomial model for each of its components. The standard error of each coefficient and its significance is also provided.

Drivers	Coefficients $\hat{\beta}$	Standard error	t-Student	P values
Intercept	1.831e+04	6.247e+03	2.932	3.413e-03
Clay	-1.284e+00	5.604e+00	-0.023	9.817e-02
Clay ²	-4.032e-01	1.888e-01	-2.136	3.279e-02
Clay ³	2.806e-04	2.118e-05	1.325	1.854e-01
Carbon	-5.541e+02	3.512e+02	-1.578	1.148e-04
Clay*carbon	1.324e+01	1.955e+00	6.771	1.720e-11
Clay ² *carbon	-1.308e-03	3.130e-04	-0.418	0.675e+00
Carbon ²	1.244e+00	5.285e+00	0.235	0.813e+00
Clay*carbon ²	-7.825e-02	1.751e-02	-4.469	8.34e-06
Carbon ³	2.663e-02	2.197e-02	1.212	0.225e+00
[H ₃ O ⁺]	-6.462e+08	3.860e+07	-1.674	9.41e-03
Clay*[H ₃ O ⁺]	2.748e+05	1.872e+04	0.147	0.883e+00
Clay ² *[H ₃ O ⁺]	1.317e+03	2.959e+03	0.445	0.656e+00
Carbon*[H ₃ O ⁺]	2.469e+07	1.434e+06	1.723	8.51e-02
Clay*carbon*[H ₃ O ⁺]	3.507e+04	3.670e+04	0.956	0.339e+00
Carbon ² *[H ₃ O ⁺]	-5.632e+04	7.146e+04	-0.788	0.430e+00
[H ₃ O ⁺] ²	5.446e+12	4.424e+11	1.231	0.218e+00
Clay*[H ₃ O ⁺] ²	-1.458e+10	9.984e+09	-1.460	0.144e+00
Carbon*[H ₃ O ⁺] ²	-1.750e+11	1.238e+10	-1.413	0.157e+00
[H ₃ O ⁺] ³	-4.264e+15	2.025e+15	-0.210	0.833e+00
Altitude	-2.292e+01	2.039e+01	-1.124	0.261e+00
Clay*altitude	2.898e-01	1.031e-02	2.811	4.987e-04
Clay ² *altitude	-6.280e-04	1.524e-04	-4.121	3.94e-05
Carbon*altitude	-1.151e+00	4.999e-01	-2.303	2.14e-03
Clay*carbon*altitude	7.013e-03	1.395e-03	5.028	5.45e-07
Carbon ² *altitude	8.390e-05	2.918e-04	0.029	0.977e+00
[H ₃ O ⁺]*altitude	3.418e+05	6.386e+04	0.535	0.592e+00
Clay*[H ₃ O ⁺]*altitude	-3.47e+02	1.496e+02	-0.232	0.816e+00
Carbon*[H ₃ O ⁺]*altitude	-3.231e+04	7.619e+03	-4.241	2.33e-05
[H ₃ O ⁺] ² *altitude	7.210e+09	5.466e+09	1.319	0.187e+00
Altitude ²	5.035e-02	1.521e-03	3.310	9.51e-04
Clay*altitude ²	-1.829e-04	3.245e-05	-5.636	2.01e-08
Carbon*altitude ²	2.450e-04	1.626e-05	1.507	0.132e+00
[H ₃ O ⁺]*altitude ²	-2.121e+01	2.622e+01	-0.081	0.933e+00
Altitude ³	-9.89e-06	4.271e-07	-2.316	2.03e-02

estimates of microbial biomass with the polynomial model. It was therefore important to determine the error on the model estimate according to the measurement errors on soil pH, carbon and clay contents (Fig. S6). Introduction of a 5% random error in pH, SOC and clay content measurements revealed that soil pH needed to be measured more accurately than SOC, altitude or clay content to reduce the error on the estimated soil microbial biomass.

3.5. Operational application of the model to diagnose soil agricultural management

To determine the accuracy of the polynomial model in diagnosing the impact of agricultural practices on soil microbial biomass, it was applied to a large set of soil samples collected over an agricultural landscape at Fénay (Constancias et al., 2015a). At the landscape scale, land use and agricultural practices were clustered into 6 categories which were discriminated first by land cover (forest vs. agricultural plots), second by soil tillage intensity (no tillage, minimum tillage, mechanical hoeing, conventional tillage), and finally by the presence of a catch crop. These clusters followed a gradient in cropping intensity and in the diversity and persistence of plant cover *i.e.*, forest (forest, no tillage, no catch crop, $n=43$); perennial crop (3 frequently mowed grasslands, 3 blackcurrant and 1 Miscanthus, $n=7$); catch crop (agricultural plot, minimum tillage, catch crop, $n=22$); minimum tillage (agricultural plot, minimum tillage, no catch crop, $n=56$); conventional tillage (agricultural plot, conventional tillage, no catch crop, $n=103$); mechanical hoeing (agricultural plot, mechanical hoeing, no catch crop, $n=33$).

Plotting of the predicted values vs. the measured values of molecular microbial biomass showed an important scatter of the points around the $y=x$ line (Fig. 4), which indicated that for a consistent number of soil samples the measured microbial biomass was either higher or lower than the predicted values. To interpret the results and establish a diagnosis of soil microbial status, we considered that predicted and measured values were similar only for the points included within the band of $\pm 20\%$ around the $y=x$ line (Fig. 4). This threshold was chosen since it corresponded to the range of uncertainty of the method of soil DNA extraction and quantification (Bourgeois et al., data not shown). It allowed three groups of samples to be distinguished. The first group consisted of the samples for which the measured values were similar to the predicted values and represented 44% of the total samples. For this group, the correspondence between the measured and the predicted values indicated that the soil microbial biomass was well predicted by the four explanatory variables taken into account in the model

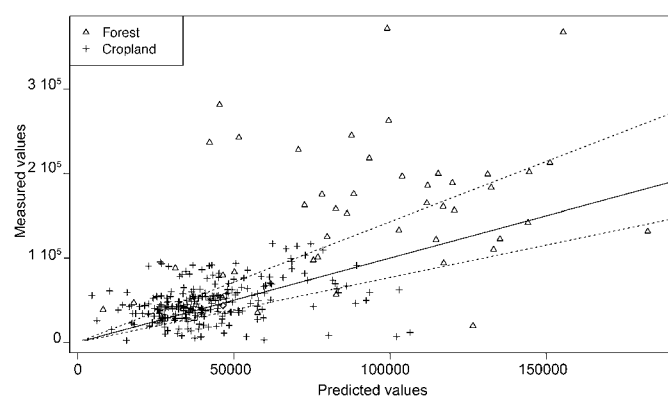


Fig. 4. Relationship between the measured and adjusted values of soil microbial biomass in the Fénay landscape. Adjusted values were derived from the third degree polynomial model. The black line represents the 1:1 line ($y=x$), dotted lines correspond to measurement uncertainty of soil microbial biomass ($\pm 20\%$), black crosses represent cropland soils and open triangles the forest soils.

(*i.e.* clay, carbon, pH, and altitude), hence suggesting no or little impact of the type of land use on the soil microbial biomass. The second and third groups consisted of the samples for which the predicted values were respectively significantly lower (19% of the total samples) or higher (37% of the total samples) than the measured values, which implied a significant impact of land use in terms of stimulation or decrease of the soil microbial biomass.

When discriminating the samples between cropped and forest soils, it was clearly apparent that microbial biomass was favored in forest soils, with 60% of the forest samples exhibiting higher measured values than predicted values (Fig. 5). This was in agreement with many other studies which reported higher microbial biomass in forest soils compared to cropped soil, mainly attributed to higher carbon content commonly occurring in forest soils (Arrouays et al., 2001). In our study however, the observed stimulation of microbial biomass might not be directly explained by soil carbon content since this is one of the explanatory variables taken into account in the model. In these soils, it is more likely that the observed stimulation is due to the improved soil structure resulting from the higher carbon content and absence of soil physical disturbance since these factors are known to be associated with the improvement of soil microbial habitats in terms of diversity and stability (Constancias et al., 2014). In addition the absence of pesticide applications such as (*i.e.* fungicides) may also contribute to the observed stimulation compared to cropped soils.

Contrastingly with forest soils, cropped soils were equally distributed between the three groups of samples, with 33%, 46% and 21% of the measured values being respectively higher, similar to, or lower than the predicted values. This indicates that, at the scale of the agricultural landscape at Fénay, soil microbial biomass was impacted either positively or negatively by cropping. Comparison of the types of agricultural managements evidenced the following gradient around the predicted values: conventional tillage = mechanical hoeing \leq predicted values $<$ minimum tillage \leq minimum tillage + catch crop $<$ forest (Fig. 5). As mentioned above, the observed discrimination between the systems cannot be directly explained by soil parameters such as carbon and clay contents, or pH since they were included as predictive variables in the model. The depletion of soil microbial biomass in systems including soil tillage or hoeing more likely results from the mechanical disruption of microbial habitats by soil disturbance (Govaerts et al., 2007; Lienhard et al., 2013). On the other hand, the preservation of soil structure through minimum tillage led to an improvement of soil microbial biomass. This increase was further enhanced when catch crops were introduced into the rotation,

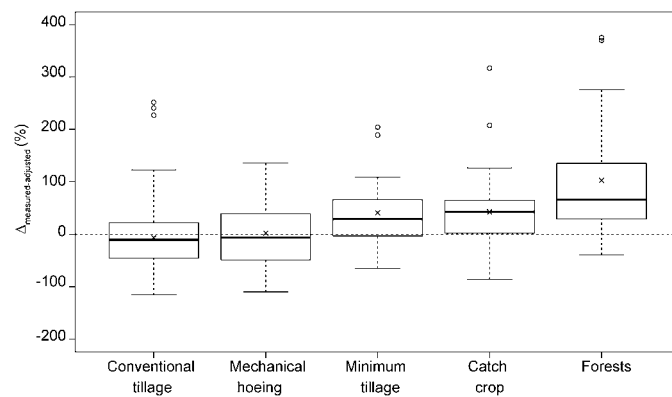


Fig. 5. Differences between predicted and measured soil molecular microbial biomass according to land management practices. For each boxplot, black cross represents the mean, bold line represents the median, sides of the box represents the first and third quartile and error bars correspond to the standard error of the mean. Open circles correspond to outliers according to the normal distribution.

thereby confirming the stimulation of microbial biomass under plant cover (Lienhard et al., 2013).

The model developed in this study is an innovative mathematical tool constituting the first operational model for assessing the microbiological status of soil in the French pedoclimatic context. Comparison of predicted and measured values provides a robust diagnosis of soil microbiological quality and its evolution under environmental pressures such as agricultural practices, industrial pollutions or more global changes. Now, similar investigations need to be conducted to develop strategies for the diagnosis of microbial diversity based on the numerous sets of massive sequencing data widely available through the international scientific community.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecolind.2015.12.004>.

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ORIGINAL RESEARCH

Contrasting spatial patterns and ecological attributes of soil bacterial and archaeal taxa across a landscape

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Introduction

Spatial patterns, based on describing the distribution of living organisms in relation to space and environmental heterogeneity, provide a key to understanding the structure and function of soil biodiversity (Martiny et al. 2006). In

Abstract

Even though recent studies have clarified the influence and hierarchy of environmental filters on bacterial community structure, those constraining bacterial populations variations remain unclear. In consequence, our ability to understand to ecological attributes of soil bacteria and to predict microbial community response to environmental stress is therefore limited. Here, we characterized the bacterial community composition and the various bacterial taxonomic groups constituting the community across an agricultural landscape of 12 km², by using a 215 × 215 m systematic grid representing 278 sites to precisely decipher their spatial distribution and drivers at this scale. The bacterial and Archaeal community composition was characterized by applying 16S rRNA gene pyrosequencing directly to soil DNA from samples. Geostatistics tools were used to reveal the heterogeneous distribution of bacterial composition at this scale. Soil physical parameters and land management explained a significant amount of variation, suggesting that environmental selection is the major process shaping bacterial composition. All taxa systematically displayed also a heterogeneous and particular distribution patterns. Different relative influences of soil characteristics, land use and space were observed, depending on the taxa, implying that selection and spatial processes might be differentially but not exclusively involved for each bacterial phylum. Soil pH was a major factor determining the distribution of most of the bacterial taxa and especially the most important factor explaining the spatial patterns of α -Proteobacteria and Planctomycetes. Soil texture, organic carbon content and quality were more specific to a few number of taxa (e.g., β -Proteobacteria and Chlorobi). Land management also influenced the distribution of bacterial taxa across the landscape and revealed different type of response to cropping intensity (positive, negative, neutral or hump-backed relationships) according to phyla. Altogether, this study provided valuable clues about the ecological behavior of soil bacterial and archaeal taxa at an agricultural landscape scale and could be useful for developing sustainable strategies of land management.

contrast to macro-organisms, the description of spatial patterns of soil microorganisms is recent, but has gained attention due to their key role in ecosystem services (Maron et al. 2011). Soil microbial communities are known to exhibit heterogeneous and structured spatial patterns at various scales ranging from the microscale (soil aggregates,

μm) to global scale (continent, $>100,000\text{ km}^2$) (Nunan *et al.* 2003; Dequiedt *et al.* 2009; Franklin and Mills 2009; Lauber *et al.* 2009; Griffiths *et al.* 2011). A large body of proof has emerged from these patterns suggesting that the abundance, diversity and assemblages of soil microbial communities are mainly determined by soil properties, plant-cover and land management, rather than by geographical barriers or climate. Thus, environmental selection (aka, niche-based process) by proximal factors would be the main process that shapes soil microbial diversity (Drenovsky *et al.* 2010; Ranjard *et al.* 2013). However, other processes based on the neutral theory have recently been shown to successfully predict nonrandom patterns of distribution (Hubbell 2001), and indicate that dispersal limitation could also significantly contribute in shaping patterns of microbial communities (Bell 2010; Stegen *et al.* 2012).

Although the processes and drivers shaping the bacterial community assembly as a whole have now been deciphered, those influencing the various bacterial taxonomic groups which constitute the community are still little known or understood. This has resulted in a lack of knowledge concerning the ecological attributes of soil indigenous bacterial taxa, which in turn limits our understanding and ability to predict community composition according to surrounding environmental conditions (Fierer *et al.* 2007; Maron *et al.* 2011) as well as our ability to link microbial diversity with soil functioning (Maron *et al.* 2011). Regarding applied ecology, this absence of knowledge is hampering the development of sustainable ecosystem management strategies based on soil microbiological resources (Levin 1992).

Spatial ecology, based on applying meta-analysis approaches under a wide range of environmental conditions, is providing useful information about the ecological attributes of indigenous soil bacterial taxa. Various authors have used spatially explicit approaches to show that the relative abundances of several bacterial taxa display contrasting patterns, thereby highlighting their distinct ecological attributes and confirming the ecological coherence of bacterial taxonomy (Philippot *et al.* 2009; King *et al.* 2010). Fierer *et al.* (2007) used meta-analysis approaches to differentiate soil bacterial taxa into ecologically meaningful categories based on the *r*-*K*-selection continuum, with *r*-strategists maximizing their intrinsic rate of growth when resources are abundant while *K*-strategists are better adapted to compete and survive when resources are limited (Pianka 1970). However, all these studies were conducted on contrasting (a priori) environmental gradients, and did not provide precise insights into the role and ecology of bacterial taxa or of the complexity of the potential ecological niches occupied by bacteria. To be able to draw conclusions about the different processes involved in community assembly, it is now crucial to decipher more precisely and with greater genericity the ecological attributes of soil microbial taxa by studying their

distribution at different scales and integrating the different environmental parameters involved such as soil types, land use, climate, geomorphology, and space.

In a previous study (Constancias *et al.* under revision), the distribution of microbial abundance and bacterial community diversity (richness, evenness and Shannon's index) was investigated across a landscape of 12 km^2 , offering an opportunity to decrypt the relative influence of soil properties and land management in shaping soil bacterial communities. The landscape, as compared to larger scales, was characterized by a smaller variability in soil properties and also by a mosaic of different types of land use constituted by forest and agricultural plots with contrasting cropping intensity. Soils ($n = 278$) were sampled within a systematic sampling grid covering the entire landscape. Soil physicochemical properties and land management characteristics were determined for each sample. Bacterial diversity was characterized by massive inventory of the 16S rRNA gene sequences amplified from soil DNA. In addition to demonstrating the heterogeneous and spatially structured distribution of microbial abundance and diversity across the landscape, variance partitioning revealed that bacterial richness is mainly driven by soil texture and pH whereas land management is a strong determinant of microbial abundance and bacterial evenness. Altogether this previous study demonstrated the relevance of the landscape scale for deciphering microbial distribution patterns and processes, and for evaluating the effects of land management strategies on soil microbial resources.

In the present study we focused on the distribution of the composition of bacterial and Archaeal communities and of the various taxonomic groups constituting the community across the landscape. The following questions were addressed: are all taxa heterogeneously distributed at this spatial scale? Do they exhibit the same patterns? Which drivers or ecological attributes characterize each bacterial and archeal taxon at this scale? To answer these questions, a geostatistical approach was used to map and describe the spatial variability of community structure and taxa, and a variance partitioning approach was applied to identify and rank the ecological attributes for each taxon. Spatial descriptors were also integrated into the analysis to better interpret their relative contributions to taxa variation across a landscape and to examine other neutral processes shaping bacterial and archeal taxa distribution.

Materials and Methods

Site, sampling strategy, and data collection

The study was carried out on a monitored landscape of 13 km^2 located in Burgundy, France (Féney, Lat: $47^{\circ}14'37''\text{N}$, Long: $5^{\circ}03'36''\text{E}$) characterized by deciduous oak-hornbeam forests (3.86 km^2) and intensive agricul-

tural croplands (9.22 km²) mainly under winter crops (winter wheat, oilseed rape) in rotation with late-sown crops (spring barley). The whole area is flat or slightly sloping, under a continental climate with a mean annual air temperature of 10.4°C and a mean annual rainfall of 762 mm (period 1968–2011). Land management practices were clustered into six categories (from forest to agricultural plots with a gradient of cropping intensity).

The sampling design, based on a square grid with spacing intervals of 215 m, covered the entire landscape and corresponded to 248 sites. It also included 30 additional sites positioned within the grid for exploring the variation over distances less than 215 m. All sites were sampled in September 2011. At each of the 278 sampling locations, five soil cores (core diameter: 5 cm; 0–20 cm depth) were randomly collected from a 4 m² area in the inter-row for agricultural sites and at least 1 m away from trees, then bulked, and 2 mm-sieved before being lyophilized at –80°C and finally archived at –40°C.

Samples were randomized before physicochemical and bacterial community characterizations to avoid any batch effect. Analyses of physicochemical properties (pH, organic carbon, total nitrogen, CaCO₃, clay, silt and sand) were carried out by the Laboratoire d'analyse des sols d'Arras of INRA (<http://www.lille.inra.fr/las>) as described in Dequiedt *et al.* (2009).

Pyrosequencing of 16S rRNA gene sequences

Soil microbial DNA was extracted using the GnsGII procedure developed by Plassart *et al.* (2012). Crude DNA was then purified using a MinElute PCR purification kit (Qiagen, Courtaboeuf, France) and quantified using the QuantiFluor staining kit (Promega, Madison, USA), prior to further investigations.

The 16S rRNA V3–V4 gene region was targeted for amplification, using primers F479 (5'-CAGCMGCYGCNGT AANAC-3') and R888 (5'-CCGYCAATTCMTTTRAGT-3') in a nested PCR strategy to add the 10-bp multiplex identifier (MID) barcode as initially described by Plassart *et al.* (2012). Equal amounts of each sample were pooled, and all further steps (adapter ligation, emPCR and 454-pyrosequencing) were carried out by Beckman Coulter Genomics (Danvers, MA) on a 454 GS-FLX-Titanium sequencer (Roche Applied Science, Indianapolis, Indiana).

The raw data sets are publicly available in the EBI database system (in the Short Read Archive) under project accession no. PRJEB5219.

Bioinformatics analysis

The generated sequences were subjected to bioinformatic analysis using the GnS-PIPE developed by the GenoSol

platform (INRA, Dijon, France) and initially described by Terrat *et al.* (2012). After an initial quality filtering step (>350 bp, no base ambiguity), reads were aligned with infernal alignments that use the secondary structure of the 16S rRNA gene (Cole *et al.* 2009) and clustered at 95% sequence similarity into operational taxonomic units (OTU). This clustering step was done using a custom PERL program that does not consider differences in homopolymer lengths, which can cause the main 454-pyrosequencing errors (Balzer *et al.* 2011). Each sample was then randomly rarefied at a sequencing depth of 10,800 quality sequences to allow rigorous comparison of the data. Community structure was characterized using weighted UniFrac distance (Lozupone and Knight 2005) calculated with the PycoGent package (Knight *et al.* 2007) on a phylogenetic tree computed using FastTree and the most abundant sequence to represent each OTU. Quality reads were used for taxonomy-based analysis by similarity approaches using USEARCH (Edgar 2010) against the corresponding Silva database (Quast *et al.* 2013).

Metadata analysis

Environmental variability of the studied landscape

The variability of soil physicochemical properties across the studied area was assessed by subjecting the data to principal component analysis (PCA). Land management practices over the entire landscape were summarized by performing a factor analysis for mixed data to define land management clusters using the *FactoMineR* package (Lé *et al.* 2008) with land use, soil tillage, crop rotation diversity (number of plant types in the crop rotation), and the pesticide treatment frequency index, as data input. These clusters followed a gradient in cropping intensity based on soil disturbance and in the diversity and persistence of plant cover that is, Forest (forest, no-tillage, no catch-crop, $n = 44$); Perennial plant cover (three frequently mown) grasslands, three blackcurrant (*Ribes nigrum*) and one Miscanthus (*Miscanthus giganteus*), $n = 7$); Catch Crop (agricultural plot, minimum tillage, catch-crop, $n = 22$); Minimum tillage (agricultural plot, minimum tillage, no catch-crop, $n = 57$); Mechanical hoeing (agricultural plot, mechanical hoeing, no catch-crop, $n = 33$) and Conventional tillage (agricultural plot, conventional tillage, no catch-crop, $n = 104$).

Ordination of microbial community structure

Differences in community structure between samples were visualized by applying the weighted UniFrac metric and Nonmetric multi-dimensional scaling (NMDS). Soil physicochemical parameters and the relative abundance of the

most dominant bacterial and archaeal phyla and Proteobacteria classes were incorporated into the analysis by vector fitting against the bi-plot ordination of community structure. Significance of the vectors was assayed by 999 permutations. Only the most significant ($P < 0.001$) vectors harboring a correlation ≥ 0.20 relative to the two NMDS axes were represented.

Interpolated mapping

A geostatistical method was used to map soil physicochemical properties (i.e., sample scores on the first three principal components of the PC Analysis conducted on physicochemical characteristics), microbial community structure (i.e., sample scores on the two axes of the NMDS analysis run on the weighted UniFrac distance matrix) and the relative abundance of the most discriminant bacterial and archaeal phyla and Proteobacteria classes. As these variables did not follow the strictly required Gaussian distribution, they were first transformed using the nonparametric rank-order (or normal scores) transformation prior to considering the spatial correlations (Juang *et al.* 2001). It is usual, in geostatistical analysis, to compute an estimate of a variogram model based on the observations which describe the spatial variation of the property of interest. This model is then used to predict the property at unsampled locations using kriging (Webster and Oliver 2007). A common requirement for variogram estimation is first to calculate the empirical (so-called experimental) variogram by the method of moments (Matheron 1965), and then to fit a model to the empirical variogram by (weighted) nonlinear least-squares. We also investigated an alternative method which uses maximum likelihood to estimate the parameters of the model directly from the data, on the assumption that this displays a multivariate normal distribution. We selected the Matérn model which can simultaneously describe several spatial processes (Minasny and McBratney 2005). The validity of the fitted geostatistical model was assessed in terms of the standardized squared prediction errors method (SSPE) using the results of a leave-one-out cross-validation. If the fitted model provides a valid representation of the spatial variation of the soil or microbial property, then these errors display a χ^2 distribution which has a mean of 1 and median 0.455 (Lark 2002). The mean and median values of the SSPE were also calculated for 1000 simulations of the fitted model to determine the 95% confidence limits. An ordinary kriging estimation was performed in the standardized-rank space and the kriging estimates were then back-transformed into the original space. The geostatistical analysis *gstat* and *GeoR* R package for variograms analysis and kriging were used (Ribiero and Diggle 2001).

Variance partitioning of community dissimilarity and of the relative abundance of bacterial and archaeal taxa

Partial regression models were conducted to estimate the contribution of physicochemical parameters, land management and space in determining variation in community dissimilarity as well as the spatial distribution of bacterial and archaeal taxa. Among the eight measured physicochemical properties, silt was removed because of co-linearity with sand and clay, and nitrogen content because of its correlation with organic content ($r = 0.92$, $P < 0.001$). In addition to the six retained physicochemical properties and the clusters summarizing land management intensity, space was characterized by using a Principal Coordinates of a Neighbour Matrix approach (PCNM). The PCNM method was applied to the geographic coordinates and yielded 76 PCNM, representing the multiple spatial scales that the sampling scheme could perceive (Ramette and Tiedje 2007). Quantitative response and explanatory data were, respectively, log-transformed and standardized to provide an approximated Gaussian and homoscedastic residual distribution. For each taxon, physicochemical and land management variables were selected by multiple regression analysis using a stepwise selection procedure, which maximized the adjusted R^2 (in order to maximize the explained variation by the model) and minimized the Akaike Information Criterion (AIC, in order to discard previously retained variables that reduced the overall predictive power). Spatial descriptors were then selected from the model residuals, in order to strictly identify the spatial autocorrelation that did not correspond to spatially structured environmental variables. These selection steps enabled us to exclude those variables that did not contribute significantly to the explained variation ($P < 0.001$), thereby limiting overfitting and problems due to co-linear variables (Ramette 2007). The respective effects of each explanatory variable, or combinations thereof, were determined by (1) partial regression for the relative abundance of taxa and (2) distance-based redundancy analysis (db-RDA, Ramette and Tiedje 2007; Bru *et al.* 2010). The statistical significance was assessed by 999 permutations of the reduced model. All these analyses were performed with R (<http://www.r-project.org/>) using the *vegan* package (Oksanen *et al.* 2011).

Results

Landscape heterogeneity of environmental parameters

The studied landscape was characterized by alkaline fine-textured soils with a mosaic of different types of land management constituted by forest (18% of the area) and agricultural

plots (82% of the area, Fig. 1A) subjected to contrasting agricultural practices. Land management was clustered into six categories to depict land management intensity (from forest to agricultural plots with a gradient of land management intensity – see Materials and Methods and Fig. 1A).

Most of the soils were silty (median 56.7%) or clayey (median 34.3%) with alkaline pH (median 8.0, Table S1). Organic carbon and total nitrogen contents were highly correlated ($r = 0.92$, $P < 0.001$) and ranged from 1.74 to 174 and 0.835 to 14.6 $\text{g} \cdot \text{kg}^{-1}$, respectively (Table S1). Soil properties were spatially structured in patches ranging from 600 to 900 m (Table S2), which reflected both the distribution of land management categories and the variations in pedological patterns (Fig. 1A and B). Due to the local chalky limestone characteristics, all soils located along the “Sans Fond” riverbed exhibited similar specific features (higher organic carbon, nitrogen and CaCO_3 contents, coarser texture and higher pH, Fig. 1) whatever the type of land management. On the other hand, samples under forest land management located at the West of the studied area and along the “Grand Fossé” riverbed

exhibited significant lower pH and higher organic carbon and nitrogen contents and C:N ratio ($P < 0.05$ in all cases, Fig. 1A, yellow patches B). Agricultural plots in the conventional-tillage and mechanical hoeing clusters were mainly situated between the villages of “Chevigny” and “Fénay” whereas most plots in the minimum tillage cluster (with or without catch crop) were found to the extreme south-west and south-east. The forests plots were mainly situated beside the two rivers (“La Sans Fond” and “Grand Fossé,” Fig. 1A).

Microbial composition variation and mapping across landscape

Pyrosequencing of 16S rRNA genes yielded a total of 5×10^6 sequences (10,800 quality sequences per sample), allowing taxonomic identification of the major bacterial and archaeal groups constituting the community in each soil sample. The NMDS ordination of Weighted UniFrac distance between samples revealed significant variation in community composition between soil samples across the

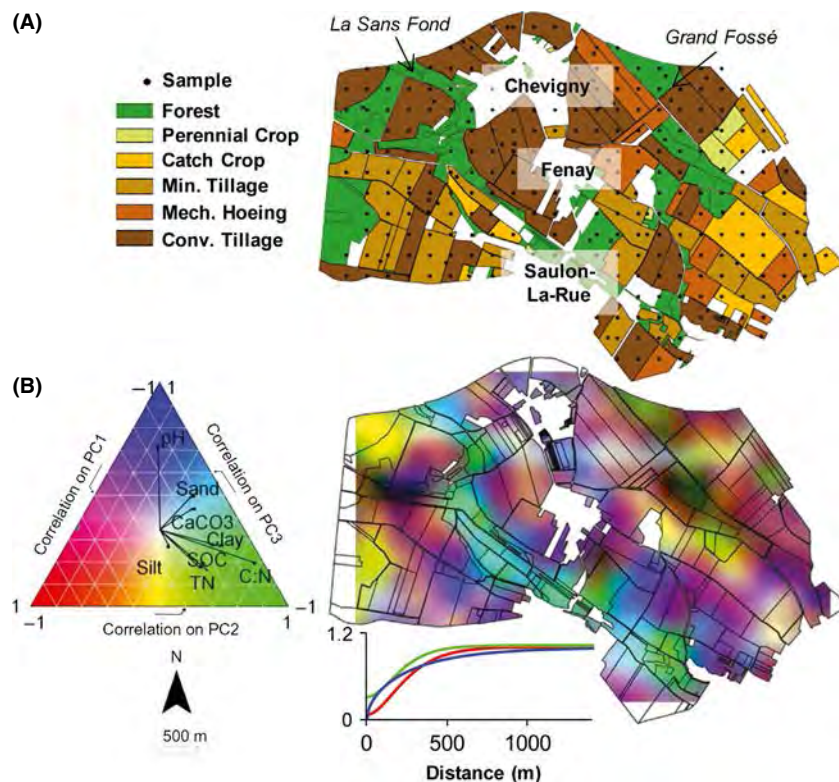


Figure 1. Maps of environmental characteristics of the Fénay landscape. (A) Maps of land management clusters including the samples location, the two rivers and the local villages in the studied area. (B) Maps of samples scores on the three-first axes of the principal component analysis conducted on the physicochemical data set: red green blue RGB color chart, Principal Component1: red, PC2: green, PC3: blue. This approach summarizes the physicochemical properties of the studied area on a single map. Correlations between axes and variables are represented to the right of the map in a triangular diagram to match the color chart. Matérn model semi-variograms of the related PC axis used to produce robust kriging are provided beside the map.

landscape (Fig. 2A). The NMDS stress of 0.09 confirmed that bacterial community could be accurately described in only two dimensions.

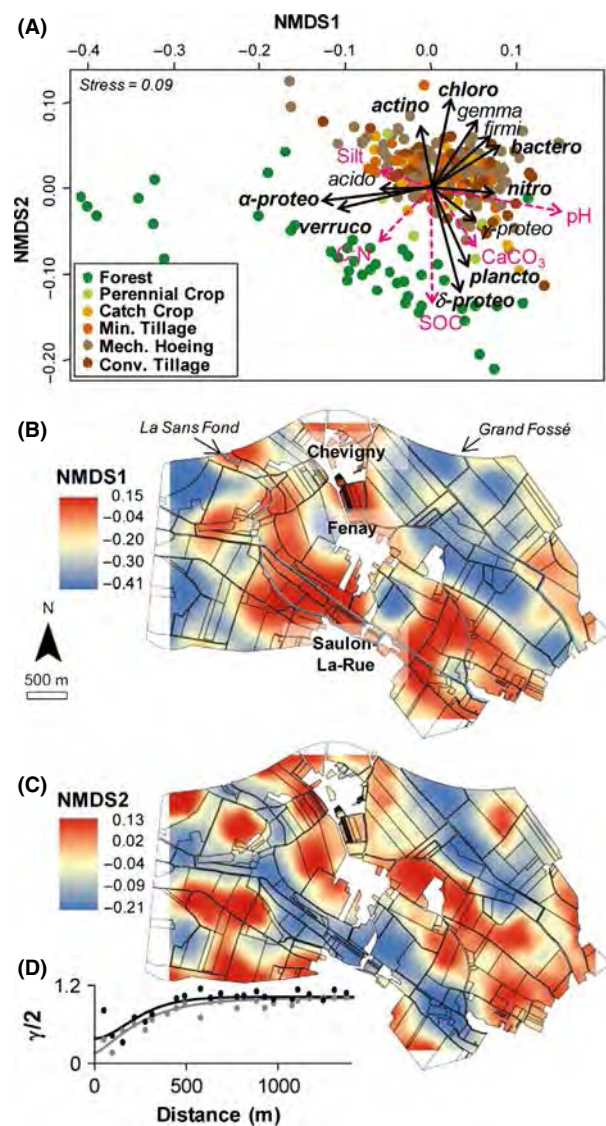


Figure 2. Nonmetric multidimensional scaling (NMDS) analysis derived from the Weighted Unifrac metric. (A) Ordination plot of the bacterial community structure. Vectors overlay were constructed based on the physicochemical properties (light red) and the relative abundance of discriminative phyla and *Proteobacteria* classes (black). Only significant correlations (≥ 0.20 with $P < 0.001$) are displayed. The angle and length of the vector indicate the direction and strength of the variable. Maps of the bacterial community structure based on the sample scores on NMDS first (B) and second dimension (C), thus, reflecting the community composition reduced to only two dimensions. The color scale to the left of each map indicates the extrapolated sample scores on the corresponding NMDS axis. (D) Semi-variograms of the transformed sample scores of NMDS1 (grey points and line for experimental and model variograms, respectively) and NMDS2 (black points and line, for experimental and model variograms, respectively).

Vector fitting of the environmental parameters against the ordination plot of NMDS revealed that bacterial composition discrimination on the first axis was mainly explained by pH ($R^2: 0.82$, $P < 0.001$), and silt content ($R^2: 0.20$, $P < 0.001$), whereas the main environmental parameters involved in discrimination on the second axis were soil organic carbon ($R^2: 0.61$, $P < 0.001$), CaCO₃ ($R^2: 0.36$, $P < 0.001$) and also silt contents ($R^2: 0.20$, $P < 0.001$) (Fig. 2A). In addition, the bacterial communities under forests strongly differed from croplands on both axes in accordance with the lower pH, higher organic carbon content and higher C:N ratio (Fig. 2A). No significant discrimination was observed in relation to the cropping intensity associated with the different agricultural land management clusters.

More precisely, the db-RDA analysis revealed that physicochemical data, land management and space explained 73% of the variation in community composition. This analysis confirmed that soil physicochemical characteristics and land management practices strongly contributed to community variation (24%, $P < 0.001$ and 7%, $P < 0.001$, respectively) and also revealed the significant marginal effect of space in shaping community variations (3%, $P < 0.001$).

Mapping of NMDS1 scores revealed a heterogeneous distribution of bacterial composition constituted by large patches with an effective range of 741 m (Fig. 2B, Table S2). The bacterial community compositions were similar at the center of the studied area (i.e., all along the “Sans-Fond” riverbed and around the “Chevigny,” “Fenay” and “Saulon-La-Rue” local villages), and contrasted with the communities located at the extreme West and at the East (i.e., around the “Grand Fossé” riverbed) of the landscape (Fig. 2B). The NMDS2 map exhibited smaller patches with a range of 574 m (Fig. 2C, Table S2) and strong variations in community composition to the West and East of the studied area (Fig. 2C). The robustness of these interpolated maps was supported by the cross validation statistics (Table S2).

The taxonomic affiliations at the phylum level, according to 16S rRNA gene sequences, revealed that the soils were generally dominated by α -Proteobacteria (mean relative abundance 23.6%, Table S1), γ -Proteobacteria (11.3%), Actinobacteria (11.2%), δ -Proteobacteria (10.8%), Bacteroidetes (8.4%), Acidobacteria (6.0%), and Firmicutes (5.5%). The bacterial and archaeal taxa involved in the bacterial community discrimination on the NMDS analysis were identified by vector fitting against the ordination plot. The main taxa explaining the community composition discrimination across this landscape were: on the first NMDS dimension, α -Proteobacteria ($R^2 = 0.78$, $P < 0.001$), Verucomicrobia ($R^2=0.49$; $P < 0.001$), Nitrospirae ($R^2 = 0.26$; $P < 0.001$) as well as δ -Proteobacteria

($R^2 = 0.76$; $P < 0.001$), Chloroflexi ($R^2 = 0.53$; $P < 0.001$), Bacteroidetes ($R^2 = 0.42$; $P < 0.001$), Planctomycetes ($R^2 = 0.39$; $P < 0.001$) on the second NMDS dimension. Forest samples were distinguished by a higher relative abundance of α -Proteobacteria, Verrucomicrobia, δ -Proteobacteria and Planctomycetes, and a lower relative abundance of Actinobacteria, Chloroflexi, Gemmatimonadetes, Firmicutes, Bacteroidetes, and Nitrospirae (Figs. 2A and S2).

Bacterial and archaeal phylum variation and mapping across landscape

The relative abundance of each bacterial phylum constituting the community on the krigged maps was interpolated by geostatistical approach. These maps evidenced a heterogeneous distribution of all the studied phylum,

supported by the cross validation statistics (Table S2), with an effective patch range between 149 and 1147 m (Fig. 3). As smaller spatial autocorrelation ranges were recorded for γ -Proteobacteria and Acidobacteria than in our usual sampling grid (i.e., <200 m, Table S2 vs. 215 m), no interpolated mapping was performed for these two bacterial taxa.

Four major patterns could be distinguished for phyla across the Fény landscape and were ranked according to patch size. α -Proteobacteria, Bacteroidetes and Nitrospirae exhibited similar and “spotty” distributions, corroborated by autocorrelation ranges around 500 m, and low ν -parameter values confirming raw spatial processes at smaller distances (Table S2). The maps of Fibrobacteres, Armatimonadetes, Gemmatimonadetes, Crenarchaeota, and β -proteobacteria across the landscape were patchier (Figs. 3 and S1) with a spatial autocorrelation range around 600 m

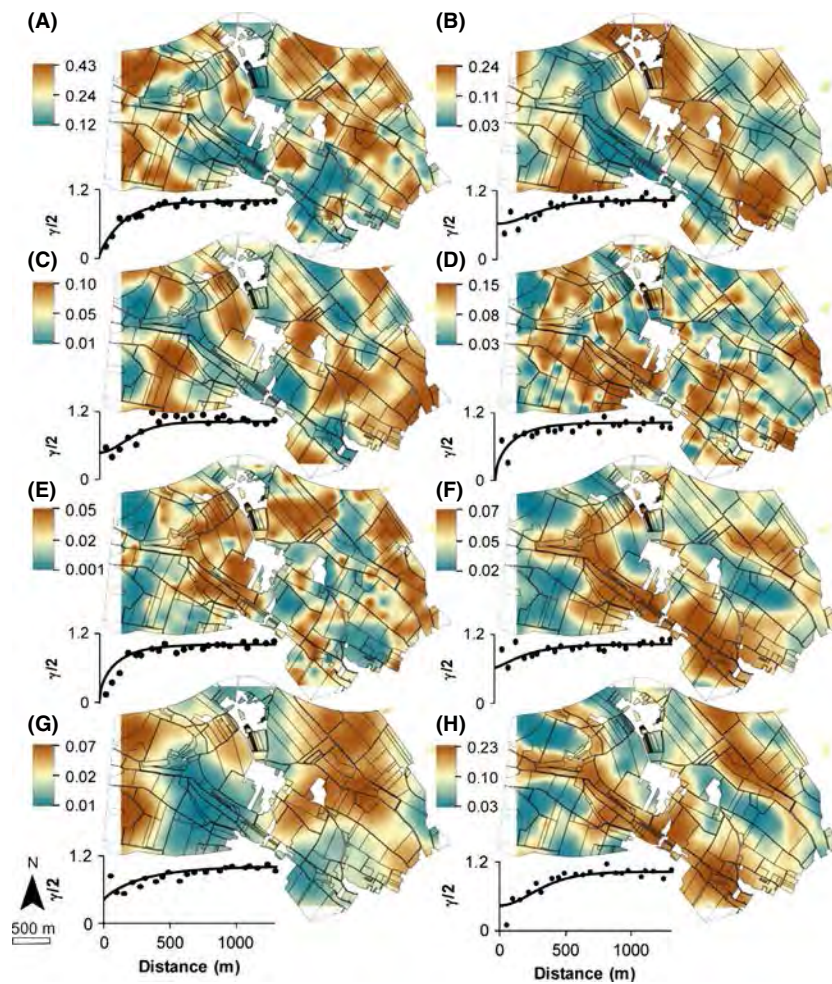


Figure 3. Maps of the relative abundance of most discriminative bacterial phyla and Proteobacteria classes across the Fény landscape according to Figure 2A. (A) α -proteobacteria; (B) Actinobacteria; (C) Chloroflexi; (D) Bacteroidetes; (E) Nitrospira; (F) Planctomycetes; (G) Verrucomicrobia and (H) δ -proteobacteria. The color scale to the left of each map indicates the extrapolated relative abundance values. Semi-variograms used to describe and model the spatial pattern are provided beside each krigged map (experimental semi-variogram; points and models; lines).

(Table S2). Planctomycetes, δ -Proteobacteria, Chloroflexi, Chlorobi, and Actinobacteria exhibited a spatial autocorrelation range around 700 m (Table S2), with high relative abundances for Planctomycetes, δ -Proteobacteria and Chlorobi, versus a lower relative abundance for Actinobacteria all along the “Sans Fond” riverbed (Figs. 3B, F, H and S1). Finally, the distributions of Firmicutes, Thaumarchaeota, Verrucomicrobia, and Elusimicrobia were relatively smooth describing large patches (autocorrelation ranges around 1000 m, Table S2). More precisely, Firmicutes and Thaumarchaeota exhibited similar spatial distributions, which contrasted with the distribution of Verrucomicrobia (Figs. 3 and S1).

Variance partitioning of bacterial and archaeal taxa distribution

A data set for soil physicochemical properties, land management and space was then used to partition the variance in taxa variation across the landscape. This approach demonstrated that between 10% and 73% of the total amount of variance could be explained according to taxa (Fig. 4). The highest amount of explained variance was observed for δ -Proteobacteria, α -Proteobacteria, Chloro-

flexi, Gemmatimonadetes, and Verrucomicrobia (from 57% to 73%, Fig. 4), whereas variations in γ -proteobacteria and Acidobacteria were weakly explained (10% and 24%, respectively, Fig. 4).

Soil physicochemical parameters were the most important predictors for 10 out of the 19 phyla studied, and explained up to 47% of the total variance (Fig. 4). On the other hand, Chloroflexi, Armatimonadetes, Gemmatimonadetes, and Firmicutes were mainly influenced by land management, which explained between 30% and 44% of their variation (Fig. 4). Interestingly, the variations in a few bacterial phyla, including Actinobacteria, β -Proteobacteria, Chlorobi, and Elusimicrobia could not be significantly explained in terms of land management (Fig. 4). Except for Planctomycetes, residual spatial autocorrelation was significantly involved in bacterial taxa variations and explained significant amounts of variance (from 2.4% to 24% Fig. 4). Moreover, only spatial parameters were involved in explaining the variation of γ -Proteobacteria and Acidobacteria (10% and 24%, respectively, Fig. 4).

The marginal effects of each parameter within the sets of soil characteristics were ranked according to the respective amounts of variance explained, and to their

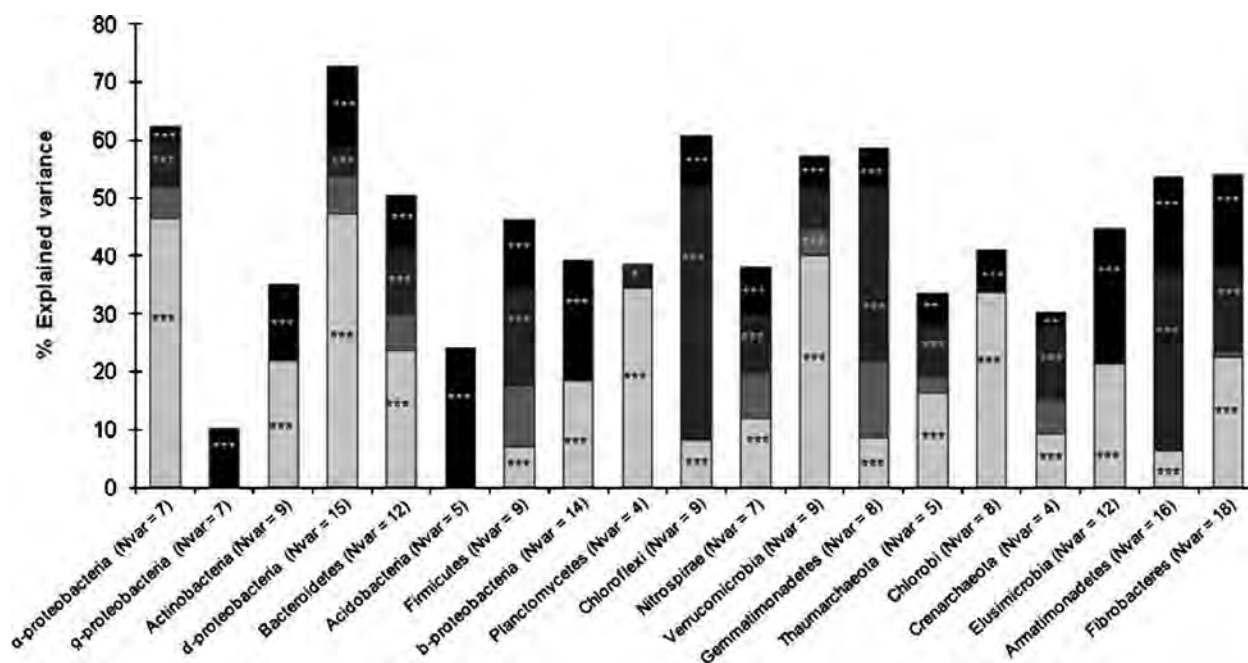


Figure 4. Partitioning of the variation of the bacterial phyla across the Fény landscape according to environmental and spatial parameters. N_{var} is the number of explanatory variables retained after selecting the most parsimonious explanatory variables (by minimizing the Akaike Information Criterion and maximizing the adjusted R^2). Bacterial phyla and *Proteobacteria* classes are ranked from the most to the least abundant. The explained variance corresponds to the adjusted R^2 values of the contextual groups of parameters (■: physicochemical characteristics, ■: land management, ■: space and ■: shared amount of variance between physicochemical properties and land management, using partial regressions). The significance level of the contribution of the sets of variables is indicated as follows; ns: not significant; $P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***. Missing values indicate that no variable of the relating group was retained in the model.

standardized estimated coefficients, which indicated a positive or negative influence on bacterial and archaeal taxa variations. Only a small number of parameters were involved in explaining the distribution of phyla belonging to β -Proteobacteria, Chlorobi, and Firmicutes, (Fig. 5), whereas a larger number of parameters were involved in determining the variation of phyla such as α -Proteobacteria, δ -Proteobacteria, Verrucomicrobia, and Fibrobacteres (Fig. 5). Soil pH contributed in explaining the variation in 14 of the 19 studied phyla and explained the highest amounts of variance (Fig. 5). More precisely, pH was positively correlated with the relative abundance of δ -Proteobacteria, Bacteroidetes, Planctomycetes, and Thaumarchaeota but negatively correlated with that of α -Proteobacteria, Verrucomicrobia, and Fibrobacteres (Fig. 5). Clay and sand contents were involved in explain-

ing variations in nine of the 19 studied phyla, but were only significant in explaining β -Proteobacteria variations through their negative influence on its relative abundance (Fig. 5). CaCO₃ content negatively impacted the variation of Actinobacteria, but positively affected that of δ -Proteobacteria, Chlorobi and Elusimicrobia (Fig. 5). Soil organic carbon content and C:N ratio were involved in a small number of phyla variations and explained small amounts of these variations.

Land management was not included in the filter-ranking due to the impossibility of determining the relative contributions of each category. However, comparison of the signs and values of the standardized estimated coefficients highlighted the contrasting influences of land management intensity on taxa variation. A positive relationship was observed between cropping intensity

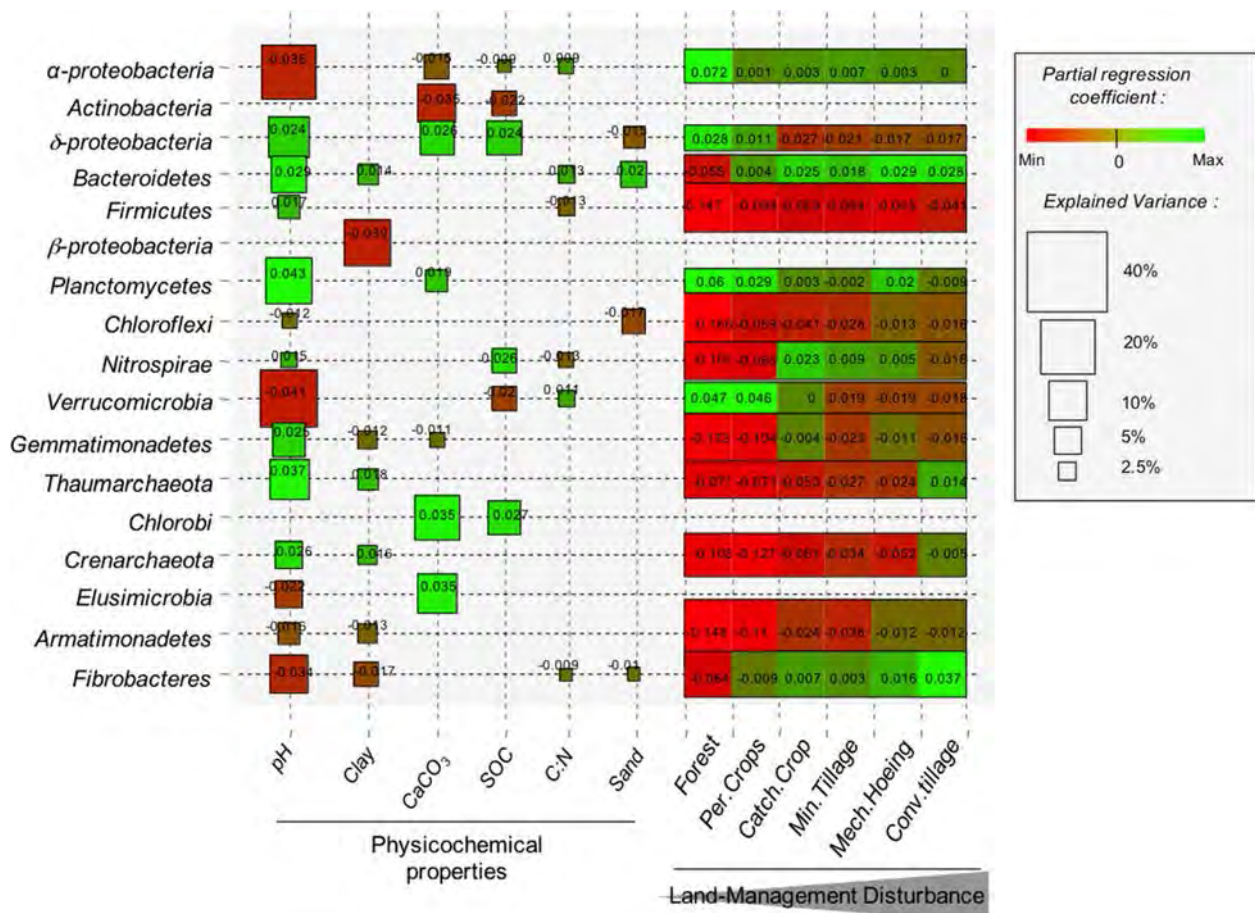


Figure 5. Contribution and effect of physicochemical and land management variables in the distribution of bacterial phyla. The respective significant contribution of each variable is represented by the height of the shape and was calculated by taking into account all other variables using partial regression models and adjusting the R^2 values. The color was scaled to depict the value of the standardized partial regression coefficients (green, positive, red negative effect). γ -Proteobacteria and Acidobacteria are not represented since no significant contribution of any physicochemical or land management variables explained their variations in the data set. Bacterial taxa are ranked according to their overall relative abundance in the data set.

(from forest to conventional tillage) and variations in bacterial and archaeal taxa belonging to Bacteroidetes, Firmicutes, Chloroflexi, Gemmatimonadetes, Thaumarchaeota, Crenarchaeota, and Fibrobacteres (Fig. 5). On the contrary, a negative influence of cropping intensity was observed for δ -Proteobacteria, Planctomycetes, and Verrucomicrobia (Fig. 5). An unusual response was observed for Nitrospira in that the response curve was hump-backed and centered on catch crop and minimum tillage (Fig. 5).

Discussion

Although spatial patterns of microbial diversity have been well documented from micro-scale (e.g., Constancias *et al.* 2014) to continental scale (e.g., Fierer and Jackson 2006) these studies did not provide significant insights into the processes and ecological attributes regulating bacterial composition and the populations constituting the whole community. Here, we focused on an agricultural landscape to determine the relative influence of land management intensity and soil physicochemical parameters on soil bacterial and archaeal community composition and populations.

First, our study provided original maps of bacterial and archaeal community composition revealing significant spatial patterns and emphasizing that microbial communities are not randomly distributed at the landscape scale as previously observed at other scales (King *et al.* 2010). Visual comparison of the patches obtained for community composition and environmental characteristics revealed significant matches suggesting a significant influence of both land management and soil characteristics. This was statistically confirmed by the variance partitioning analysis, which also revealed that space explained a significant part of soil microbial community variation. This result implies that neither deterministic processes (environmental selection) nor neutral processes (dispersal limitation) are exclusive in explaining community composition variation (Martiny *et al.* 2011; Ranjard *et al.* 2013). A similar observation was reported for macroorganisms (Martiny *et al.* 2006), and for microorganisms at territorial or continental scales (Martiny *et al.* 2011; Ranjard *et al.* 2013) and more recently, it has been shown that models based on the neutral theory are able to predict the distribution patterns of microorganisms (Sloan *et al.* 2006; Woodcock *et al.* 2007).

Among the soil properties, pH was one of the most significant drivers of bacterial composition. Fierer and Jackson (2006) suggested that pH imposes significant and direct physiological stress on bacterial cells, selecting the best-adapted ones. The primary role of pH on bacterial community diversity and composition has been demon-

strated in numerous studies over the past decade (e.g., Fierer and Jackson 2006; Rousk *et al.* 2010; Shen *et al.* 2013). In our case, even if pH exhibited weak variability (mean of 7.7, with a median of 8.0) across the landscape, it mainly influenced bacterial community variations. On the other hand, this small pH variation made it possible to show that texture, organic carbon content and C:N are also important drivers of bacterial community structure. Soil texture has been shown to control habitat number and diversity in terms of hosting and protecting microbial communities against several abiotic and biotic stresses, including desiccation and predation from protozoa, for example (Ranjard and Richaume 2001). Covariations between bacterial community composition and organic carbon content and C:N ratio confirmed the influence of nutrient quantity and quality on microbial community composition (Dequiedt *et al.* 2009). This covariation could result from competition between bacterial populations for different types of soil organic matter according to their copiotrophic/oligotrophic attributes (Bernard *et al.* 2007; Fierer *et al.* 2007).

Organic content, C:N ratio and pH are strongly impacted by land management and especially agricultural practices (Arrouays *et al.* 2001). This is coherent with the strong discrimination of bacterial communities observed between forest and croplands, which exhibited differences in soil characteristics. The influence of agricultural land management, which had been separated into different clusters based on cropping intensity and soil disturbance by tillage, did not reveal any significant discrimination of bacterial community composition between these clusters. This result is not in agreement with previous experimental trials where tillage intensity was shown to be an important driver of soil microbial communities (Acosta-Martínez *et al.* 2010; Lienhard *et al.* 2013). Therefore, soil characteristics rather than agricultural practices have a stronger influence on bacterial community composition at a landscape scale and only important modifications in land management type would impact bacterial community composition (Lauber *et al.* 2008; Kuramae *et al.* 2012).

By characterizing the distribution of bacterial and archaeal taxonomic groups at the landscape scale, we were able to compile, for the first time, original maps for the 19 most abundant phyla constituting the community. These maps revealed a heterogeneous and spatially structured distribution for all taxa except the Acidobacteria and γ -Proteobacteria phyla. The absence of significant spatial patterns for these two taxa might be partly due to the low pH variability across the landscape as pH is known to be an important driver for them (Lauber *et al.* 2008; Lauber *et al.* 2009; Nacke *et al.* 2011). It is interesting to note the contrasting distribution patterns of each

bacterial taxon across the landscape, with patches ranging from 493 to 1147 m depending on the taxon. Different types of distribution patterns, characterized by large, medium or small patch size, could be distinguished at this scale. Bacterial taxa such as Bacteroidetes, Nitrospira, and α -Proteobacteria exhibited spatial patterns characterized by small patches (about 500 m), which partly matched with the distribution of land management types across the landscape. Fibrobacteres, Armatimonadetes, Gemmatimonadetes, Crenarchaeota, and β -Proteobacteria were distributed in larger patches (around 600 m), which matched both the distribution of land management clusters and physicochemical characteristics. Contrastingly, the spatial patterns of taxa including Chlorobi, Actinobacteria, Planctomycetes, and δ -Proteobacteria were characterized by an intermediate patch size (about 700 m) which matched with soil characteristics and geomorphology, especially in the case of the "Sans fond" river location. Finally, taxa belonging to Thaumarchaeota, Verrucomicrobia, Elusimicrobia, and Firmicutes exhibited larger patches across the landscape (about 1000 m), which also matched with variations in soil characteristics and geomorphology. These contrasting patterns suggest that different drivers contrastingly shape bacterial taxa across the landscape. It also suggests that bacterial taxa might be differentially influenced by neutral processes (i.e., dispersal capabilities).

Variance partitioning analysis of bacterial and archaeal taxa variation revealed that soil physicochemical characteristics and land management mainly contributed in explaining the spatial distribution of 16 of the 19 taxa. This suggests that the main process shaping the distribution of bacterial and archaeal taxa across the landscape is environmental selection determined by physicochemical properties and land-use. Repeated reports of the strong influence of local soil environmental heterogeneity had led to the conclusion that selection was the only process shaping soil microbial communities (Fierer and Jackson 2006; Rousk *et al.* 2010). Interestingly, we systematically recorded a significant contribution of space in explaining the distribution of bacterial and archaeal taxa (except for Planctomycetes), which suggests that dispersal may also contribute to producing the observed patterns. However, demonstrating the influence of a dispersal process in shaping the distribution of soil microbial communities and populations is neither easy nor frequent in microbial ecology with few studies using appropriate sampling designs and modeling approaches (Hanson and Fuhrman 2012). In our case, the contrasting contribution of space depending on the taxa could result from different dispersal capabilities, which would include their abilities for passive dispersal and to successfully settle in locations characterized by contrasting environmental conditions

(Hanson and Fuhrman 2012). This differential contribution of space could also result from mass effects with populations being maintained at particular locations by the constant emigration of individuals from distant hot-spots (Leibold *et al.* 2004). This could be especially important for phyla with spatial patches outside the range of soil physicochemical characteristics and land management practices (e.g., Nitrospirae, Bacteroidetes, Firmicutes and Elusimicrobia). On the other hand, the relatively poor impact of space in determining the distributions of bacterial and archaeal taxa belonging to α -Proteobacteria, Planctomycetes, Crenarchaeota, and Verrucomicrobia could reflect the weak impact of dispersal-mediated processes. This is in agreement with the size of the patch, which matches with physicochemical variability across the landscape.

Unsurprisingly, pH emerges as the filter exhibiting the most important correlation with the distribution of most of the phyla, thus confirming its strong influence on the community composition as a whole. α -Proteobacteria, δ -Proteobacteria, Planctomycetes, and Verrucomicrobia were strongly correlated with soil pH (both positively and negatively). The acidophilic attributes of some genera belonging to α -Proteobacteria and Verrucomicrobia and the basophilic attributes of some genera belonging to Planctomycetes and δ -Proteobacteria are coherent with the correlation between these taxa and pH reported in recent studies (Nacke *et al.* 2011). Soil texture, represented by clay or sand contents, was the second most important soil driver for β -Proteobacteria, Bacteroidetes, and Chloroflexi. This suggests that some taxa are better adapted to live in less protected and oligotrophic habitats represented by coarse textured soils whereas others live in more protected and copiotrophic habitats represented by fine textured soils (Dequiedt *et al.* 2009; Constancias *et al.* 2014). More precisely, β -Proteobacteria were negatively influenced by clay content indicating that coarse textured soils are more favorable habitats for this taxon. These observations confirmed the affinity of some genera belonging to β -proteobacteria and Bacteroidetes for a disturbed environment and matched with their ecological attributes as r-strategists (Cleveland *et al.* 2007). Soil organic carbon content and C:N ratio, representing trophic quantity and quality, were less shared drivers of the bacterial and archaeal taxa and explained smaller amounts of their variation. This contrasts with Fierer *et al.* (2007), who demonstrated experimentally that most of the bacterial phyla could be simply described according to their copiotrophic and oligotrophic attributes. This discrepancy could result from the low variations in soil organic content and C:N ratio that occurred across the studied landscape. Nevertheless, the spatial distribution of δ -Proteobacteria, Chlorobi and Actinobacteria was mainly

influenced by soil organic content. More precisely, δ -Proteobacteria and Chlorobi were positively influenced whereas Actinobacteria was negatively affected by trophic quantity, thus confirming the respective copiotrophic and oligotrophic behaviors of some genera belonging to these phyla (Cleveland *et al.* 2007; Pascault *et al.* 2013).

The confrontation of soil bacterial and archaeal taxa variation with land use revealed that α -Proteobacteria, Fibrobacteres, and Bacteroidetes phyla were strongly impacted by a coarse level of land use discrimination (forest vs. croplands). These observations confirmed recent studies which highlighted a greater relative abundance of Fibrobacteres, Bacteroidetes, and a lower relative abundance of α -Proteobacteria in agricultural soils as compared to forest ecosystems (Jangid *et al.* 2008; Nacke *et al.* 2011; Shange *et al.* 2012). Similarly, the distributions of δ -Proteobacteria, Planctomycetes, Verrucomicroba, and Gemmatimonadetes were impacted by an increasing cropping intensity represented by crops versus forest and perennial crops. Planctomycetes and δ -Proteobacteria, which have been described as *K*-strategists, (Buckley *et al.* 2006; Pascault *et al.* 2013) might have an advantage under less disturbed environmental conditions. In the Fénay landscape, the catch crop mainly consisted in leguminous plants that could explain the observed ecological optimum of Nitrospirae, which includes taxa known to interact with plant communities. Bacterial and archaeal taxa including Bacteroidetes, Thaumarchaeota, Crenarchaeota, Armatimonadetes, and Fibrobacteres exhibited their ecological optima at the highest level of land management disturbance, represented by conventional tillage. Bacteria belonging to the Bacteroidetes phyla have been recently described as *r*-strategists and stress resistant which could explain their affinity for highly disturbed soil environments (Eilers *et al.* 2010). However, Thaumarchaeota, Crenarchaeota, Armatimonadetes, and Fibrobacteres are usually pooled as minor taxa (<1%), and therefore, to date, we do not possess any significant knowledge about their ecological attributes. Nevertheless, our study suggests that they can be considered as *r*-strategists.

Altogether, by studying bacterial community composition and taxa distribution at a landscape scale, we evidenced that the distribution of each taxon, as well as the community composition as a whole, is heterogeneous and spatially structured. The results of our study also emphasize that environmental selection may not be the only process that explains patterns of soil microbial community distribution. The selection process results from soil physicochemical filters (pH, texture and nutrient status), to a large extent, but also from disturbance intensity arising from human activities. Even though our study did not directly demonstrate that the influence of space was

exclusively due to dispersal limitation of the populations constituting the community, our data would support this hypothesis. In addition, a spatial approach was used to complete and define new ecological attributes for most of the taxa identified. Further investigations should now be devoted to the spatial patterns of fungal communities to fully depict the mechanisms and drivers of soil microbial biodiversity, and a more thorough analysis of the link with soil functioning.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary statistics of sample characteristics ($n = 278$).

Table S2. Geostatistics and cross-validation parameters of the physicochemical properties, bacterial community composition and bacterial population.

Figure S1. Additional maps to Figure 3 displaying the relative abundance of bacterial *phyla* and *Proteobacteria* classes across the Fény landscape. The color scale to the left of each map indicates the extrapolated relative abundance values.

ORIGINAL RESEARCH

Mapping and determinism of soil microbial community distribution across an agricultural landscape

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Abstract

Despite the relevance of landscape, regarding the spatial patterning of microbial communities and the relative influence of environmental parameters versus human activities, few investigations have been conducted at this scale. Here, we used a systematic grid to characterize the distribution of soil microbial communities at 278 sites across a monitored agricultural landscape of 13 km². Molecular microbial biomass was estimated by soil DNA recovery and bacterial diversity by 16S rRNA gene pyrosequencing. Geostatistics provided the first maps of microbial community at this scale and revealed a heterogeneous but spatially structured distribution of microbial biomass and diversity with patches of several hundreds of meters. Variance partitioning revealed that both microbial abundance and bacterial diversity distribution were highly dependent of soil properties and land use (total variance explained ranged between 55% and 78%). Microbial biomass and bacterial richness distributions were mainly explained by soil pH and texture whereas bacterial evenness distribution was mainly related to land management. Bacterial diversity (richness, evenness, and Shannon index) was positively influenced by cropping intensity and especially by soil tillage, resulting in spots of low microbial diversity in soils under forest management. Spatial descriptors also explained a small but significant portion of the microbial distribution suggesting that landscape configuration also shapes microbial biomass and bacterial diversity.

Introduction

Soil microorganisms are the most abundant and diverse living organisms on earth and are key players in the biogeochemical cycles. However, the environmental factors shaping soil microbial community abundance and assembly are still unclear, which limits our understanding of the role of soil biodiversity in ecosystem services (Gardi *et al.* 2009). Since the 18th century, ecologists studying macroorganisms have often used spatial approaches to better understand the processes and filters which drive the magnitude and the variability of biodiversity (Martiny *et al.* 2006). More recently, microbial ecologists have found that these approaches can also be applied to soil microorganisms (Ettema and Wardle 2002). Consequently, the number of soil microbiology studies integrating a spatial dimension has increased considerably during the last decade. These studies have systematically demonstrated a significant spatial structuring of microbial communities over multiple spatial scales (i.e., that the high spatial variability/heterogeneity of microbial community characteristics is not randomly distributed in space), such as: the microscale (from μm^2 to mm^2 , Nunan *et al.* 2003), plot scale (from m^2 to hundreds of m^2 ; Rousk *et al.* 2010), regional scale (from km^2 to hundreds of km^2 , Dequiedt *et al.* 2009; Drenovsky *et al.* 2010) and global scale (ca. $>100\,000\text{ km}^2$; Dequiedt *et al.* 2011; Griffiths *et al.* 2011; Fierer and Ladau 2012; Serna-Chavez *et al.* 2013). All these scales are relevant to better understand the ecology of soil microorganisms and the determinism of their diversity as they represent the multiple levels of spatial heterogeneity in the soil matrix, climatic conditions, geomorphology, and land use that drives soil microbial diversity (Ettema and Wardle 2002).

Even if the sets of environmental variables were not always completely similar among the studies at the different spatial scales, each of them allowed the identification of environmental filters shaping soil microbial communities. On a broad scale, environmental filters involved in the distribution of microbial communities were identified as soil type, with a significant effect of pH, carbon content and soil texture, as well as of additional factors such as land use and climatic conditions (Fierer and Jackson 2006; Bissett *et al.* 2010; Pasternak *et al.* 2013). At the soil microscale, factors such as porosity (Chenu *et al.* 2001) conditioning carbon substrate and nutrients availability as well as the level of protection of aggregates for microorganisms to surrounding perturbations (Constancias *et al.* 2013), were identified as drivers of microbial community variation between the different microhabitats. At the intermediate plot scale, proximal factors such as pH (Rousk *et al.* 2010), organic carbon content (Saetre and Bååth 2000), texture, and land management (Philippot *et al.* 2009) have been highlighted as important drivers. Altogether, these studies

suggested that although similar environmental drivers are involved in shaping microbial communities at every scale, particular filters may have a significant influence at a particular scale. In this context, it is now crucial to investigate an up scaling approach and provide a generic response to the question: which filter for which scale?

Considering the added complexity of shaping soil microbial diversity while up scaling, a gap remains in our knowledge of community distribution at the landscape scale, that is, intermediate between the plot and territory scales. This scale is relevant since it may integrate a strong variability in soil types potentially close to that of a region and because it is the scale of human activities at which land use and agricultural practices are integrated. Microbial investigations at this particular spatial scale are rare and have focused on particular homogeneous ecosystems in terms of land management. Zinger *et al.* (2011) focused on Alpine natural ecosystems to decipher the influence of plant cover, soil physicochemistry and space in determining soil microbial communities. Other studies focused on an agricultural landscape, but were limited to a restricted mosaic of experimental plots and did not integrate landscape variability or spatial configuration (Enwall *et al.* 2010; Wessén *et al.* 2011). Altogether, they have highlighted the need for investigations on a landscape scale to better understand the impact of land management versus soil physicochemical characteristics on indigenous microbial communities. Landscape is also the scale for human activities and decision makers, and a deeper understanding of the relative influence of land use and habitat heterogeneity on below ground soil diversity could be helpful to formulate management strategies for a sustainable land use.

The present study was designed to map and characterize the spatial variation of the soil microbial community across a landscape and to rank the environmental and land use filters influencing this distribution. The studied landscape consisted of forest and arable plots under various types of agricultural management. Soils ($n = 278$) were sampled within a systematic sampling grid (spacing of 215 m) covering the entire landscape (13 km^2). Physicochemical characteristics and the type of land management were precisely referenced for each soil. Soil molecular microbial biomass was determined from the DNA yield of each soil sample (Dequiedt *et al.* 2011) and bacterial diversity by massive inventory of the 16S rRNA gene sequences amplified from this soil DNA. Geostatistical approach was used to explain the spatial variability in microbial abundance and diversity and to provide prediction maps. The relative contributions of land management, soil physicochemical characteristics, and space in determining microbial abundance and bacterial diversity distribution were identified and ranked by variance partitioning. We hypothesized that land management, especially agricultural practices, would be the main drivers of microbial abundance

and diversity at the landscape scale, due to smaller variations of soil physicochemical characteristics than at wider scales. Spatial descriptors were also integrated into the analysis to better decipher their relative contributions to community variation across landscape and to consider other neutral processes in community distribution.

Experimental Procedures

Site description, sampling strategy, and data collection

The study was carried out on a monitored landscape covering 13 km² in Burgundy (France, Lat: 47°14'N, Long: 5°03'E), characterized by oak hornbeam deciduous forests (3.86 km²) and intensive agricultural croplands (9.22 km²). The site is under continental climate, with a mean annual air temperature of 10.4°C and a mean annual rainfall of 762 mm (period 1968 – 2011). The whole area is situated on deep calcisol (IUSS Working Group, WRB 2006) of mainly silty or silty clay texture and is slightly sloping. Croplands were planted with winter crops (winter wheat, oilseed rape) in rotation with late sown crops (spring barley). Crop species and management practices were recorded from 2004 to 2011 over the whole study area.

The sampling design covers the entire landscape and is based upon a square grid with spacing of 215 m which corresponds to 248 sites. It also includes 30 additional observations positioned randomly within the grid, which permit exploration of the variation over distances less than 215 m (10–100 m from the closest site). All sites were sampled in September 2011. At each site, five soil cores (core diameter: 5 cm; 0–20 cm depth) were collected on a surface of 4 m² at inter row for agricultural sites and at least 1 m away from trees, then bulked and sieved through 2-mm mesh. Samples were lyophilized at –80°C and stored at –40°C in the soil conservatory of the GenoSol platform (http://www2.dijon.inra.fr/plateforme_genosol). Samples were randomized prior to analysis to avoid batch effects. Physicochemical analyses (pH, organic carbon, total nitrogen, CaCO₃ and texture) were performed as described by Dequiedt *et al.* (2011). Soil organic carbon was determined by loss on ignition method (<https://www6.lille.inra.fr/las/Methodes-d-analyse/Sols/04.-Carbone-Azote-Matieres-Organiques/SOL-0402-Perte-au-feu-a-1100-C.>).

Molecular characterization of soil microbial communities

Soil DNA extraction, quantification and purification

DNA was extracted and purified from the 278 soil samples using the GnS-GII procedure as described by Plassart *et al.*

(2012). Crude DNA extracts were quantified by agarose gel electrophoresis stained with ethidium bromide and using calf thymus DNA as standard curve, reported to be reliable for estimating microbial biomass in Dequiedt *et al.* (2011). Crude DNA was then purified using a MinElute gel extraction kit (Qiagen, Courtaboeuf, France) and quantified using Quanti-Fluor staining kit (Promega, Madison, Wisconsin, USA), prior further investigations.

PCR amplification and pyrosequencing of 16S rRNA gene sequences

Amplification targeted the 16S rRNA V3-V4 gene region using primers F479 and R888 and a nested PCR strategy to add a 10-bp multiplex identifier (MID) barcode, as initially described by Plassart *et al.* (2012). Equal amounts of each sample were pooled, and all further steps (adapter ligation, emPCR and 454-pyrosequencing) were carried out by Beckman Coulter Genomics (Danvers, MA, <http://www.beckmangenomics.com/>) on a 454 GS-FLX-Titanium sequencer (Roche, Basel, Switzerland). The raw data sets are publicly available in the EBI database system (in the Short Read Archive) under project accession no. PRJEB5219.

Bioinformatics sequence analysis

The bioinformatics analyses were performed using the GnS-PIPE at the GenoSol platform Terrat *et al.* (2012). Sequences obtained after an initial quality filtering step (>350 bp, no base ambiguity), were aligned with Infernal alignments using a secondary structure of the 16S rRNA gene (Cole *et al.* 2009), and clustered at 95% sequence similarity into operational taxonomic units (OTU). Clustering was done with a custom PERL program that does not take into account differences in homopolymer length, which can constitute one of the major 454 sequencing errors (Balzer *et al.* 2011). Procedure details are provided in Table S1. A subsample of 10,800 quality sequences for each sample was randomly selected to allow rigorous comparison of the data. Bacterial diversity was characterized by OTU richness, evenness, and Shannon index (Haegeman *et al.* 2013).

Metadata analysis

Clustering of land cover and agricultural practices into land management categories

In order to summarize the land management practices over the entire landscape, a factor analysis for mixed data was used to define land management clusters using the *FactoMineR* package (Lê *et al.* 2008) with input data such as land use, soil tillage, crop rotation diversity (number

of plant types in the crop rotation), pesticide treatment frequency index.

Interpolated mapping

A geostatistical method was used to map physicochemical data and microbial communities and to characterize their spatial variations. As the studied variables do not follow a required Gaussian distribution, they were first transformed using the non parametric rank order (or normal scores) transformation prior to considering the spatial correlations (Juang *et al.* 2001). Conventionally in geostatistical analysis, an estimate of a variogram model is computed based on the observations which describe the spatial variation of the property of interest. This model is then used to predict the property at unsampled locations using kriging (Webster and Oliver 2007). A usual method for variogram estimation is first to calculate the empirical (so called experimental) variogram by the method of moments (Matheron 1965), and then to fit a model to the empirical variogram by (weighted) nonlinear least squares. We investigated also an alternative method which uses maximum likelihood (ML) to estimate parameters of the model directly from the data, on the assumption that it is a multivariate normal distribution. We retained the Matérn model which can describe various spatial processes (Minasny and McBratney 2005). The validity of the fitted geostatistical model was assessed in terms of the standardized squared prediction errors (SSPE) using the results of a leave one out cross-validation. If the fitted model is a valid representation of the spatial variation of the soil or microbial property, then these errors have a χ^2 distribution which has a mean of 1 and median 0.455 (Lark 2002). The mean and median values of the SSPE were also calculated for 1000 simulations of the fitted model to determine the 95% confidence limits. The ordinary kriging estimation was performed in the standardized rank space and then the kriging estimates were back transformed into the original space. We used the geostatistical analysis *gstat* and *GeoR* R package for variograms analysis and kriging (Ribiero and Diggle 2001).

Variance partitioning

The relative contributions of soil physicochemical parameters, land management (Fig. 1), and space in shaping the patterns of soil microbial abundance and bacterial diversity were estimated by variance partitioning. A Principal Coordinates of a Neighbour Matrix approach (PCNM) was used to describe and identify the scales of spatial relationship between samples (Dray *et al.* 2006). This PCNM method was applied to the geographic coordinates and yielded 76 PCNM with significant Moran index

($P < 0.001$), representing the spatial scales that the sampling scheme could perceive (Ramette and Tiedje 2007). The spatial neighborhood described by each PCNM was determined from Gaussian variogram models (Bellier *et al.* 2007). All quantitative (response and explanatory) data were standardized in order to have an approximated Gaussian and homoskedastic residual distribution. To determine the environmental parameters significantly shaping bacterial communities, a stepwise selection procedure was first applied to all physicochemical and land management variables by maximizing the adjusted r^2 while minimizing the Akaike Information Criterion (Ramette 2007). Spatial descriptors were then selected from the model residuals (Brocard *et al.*, 2004). These selection steps were done to limit over fitting and to exclude co linear variables (Ramette 2007). The respective amounts of variance (i.e., marginal and shared) were determined by canonical variation partitioning and the adjusted r^2 with RDA (Ramette 2007) for microbial biomass, bacterial richness, evenness, and Shannon's diversity index. The statistical significance of the marginal effects was assessed from 999 permutations of the reduced model. All these analyses were performed with R using the *vegan* package (Oksanen *et al.* 2011). All these analyses were performed with the R free software (<http://www.r-project.org/>).

Results

Landscape variability and distribution of environmental characteristics

Most soils (70%) in the studied landscape were silty (median 56.7%) or clayey (median 34.3%) with alkaline pH (median 8.0, Table 1). Organic carbon and total nitrogen contents were highly correlated ($r^2 = 0.92$, $P < 0.001$) and ranged from 1.74 to 174 and 0.835 to 14.6 g.kg^{-1} , respectively (Table 1). Geostatistical mapping of the environmental variables revealed a heterogeneous distribution of soil characteristics across the landscape, which was spatially structured (Fig. 1). High values of soil organic carbon content were systematically found under forest and in agricultural plots beside the “*La Sans Fond*” and “*Grand Fossé*” rivers (Fig. 1A). Acidic soils were mainly located at the north east of “*Fényay*” village and in the western part of the studied area. Alkaline soil zones were found near the “*La Sans Fond*” river (Fig. 1B), together with high sand and CaCO_3 contents (Fig. 1C–E). The validity of the spatial predictions of soil characteristics was confirmed by the results of the cross-validation. All the indicators (median and mean of the SSPEs) fall within the 95% confidence intervals (Table S2). The fitted models gave effective ranges from 611 to 839 m, depending on the soil parameters (Fig. 1, Table S2), indicating

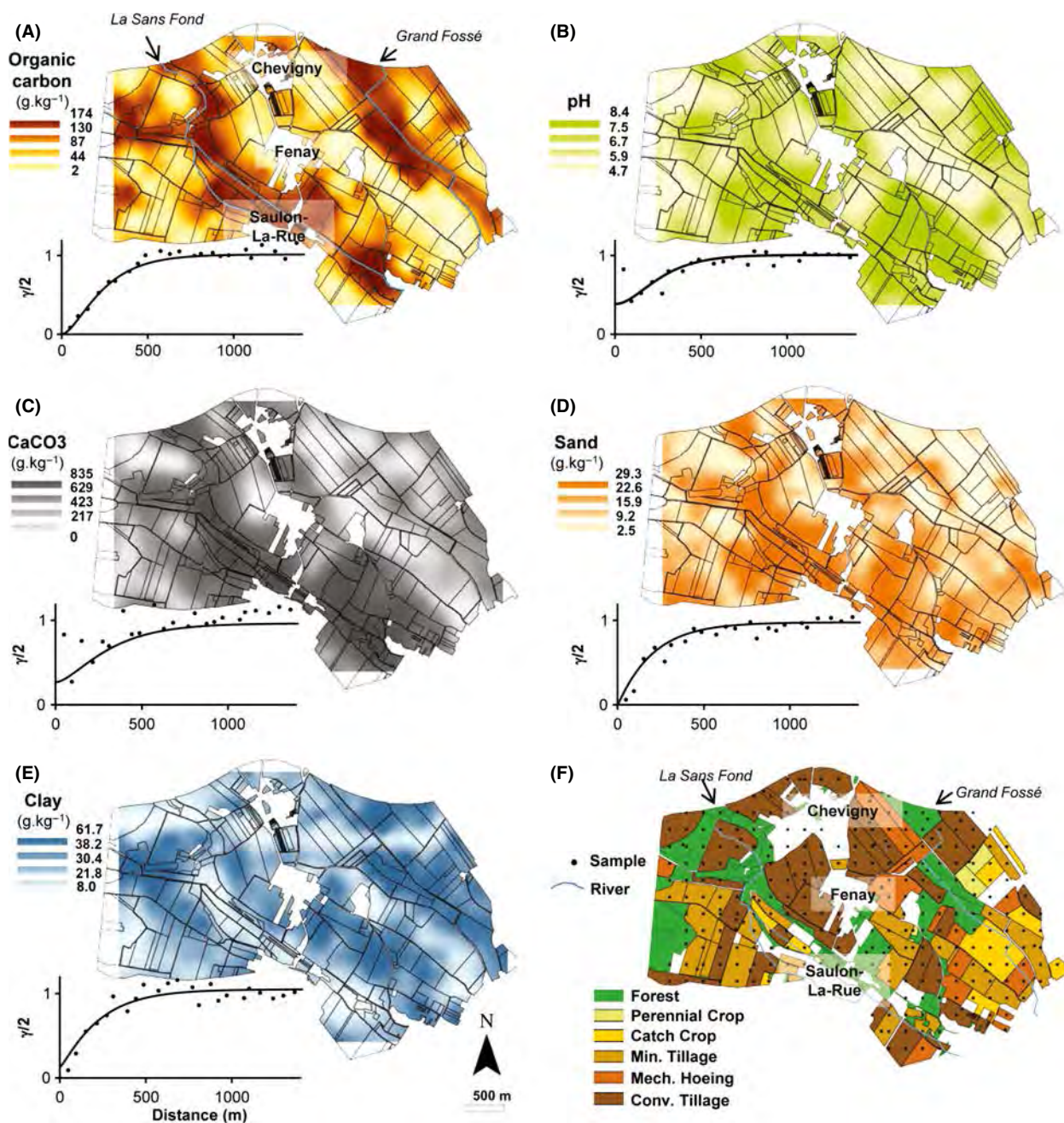


Figure 1. Maps and variogram soil and land use characteristics observed at the scale of the Fényay Landscape. Map of (A) soil organic carbon content, (B) soil pH, (C) CaCO_3 , (D) sand content, (E) clay content, and (F) land management clusters. Points indicate the sampling locations. Min., minimum; mech., mechanical; conv., conventional. For each kriged map the color scale to the left of each map indicates the extrapolated values expressed as g.kg^{-1} of sample excepted for pH. Points represent the experimental variogram, continuous lines the Matérn models fitted by maximum likelihood method. Geostatistics and cross-validation parameters are provided in Table S2.

that all soil characteristics were spatially structured in patches of several hundreds of meters.

Land use and agricultural practices were clustered into six categories (from forest to agricultural plots with a gradient of cropping intensity; see materials and methods)

and mapped across the landscape. Six clusters were identified and discriminated first by land cover (forest vs. agricultural plots), secondly by soil tillage intensity (no tillage, minimum tillage, mechanical hoeing, conventional tillage) and finally by the presence of a catch crop. The

Table 1. Summary statistics of soil characteristics ($n = 278$).

	Mean (SD)	Median	[min; max]
Physicochemical			
Organic carbon (g.kg^{-1})	21.9 (15.8)	17.1	[1.7; 174]
Total nitrogen (g.kg^{-1})	2 (1.3)	1.6	[0.8; 14.6]
C:N ratio	10.7 (1.6)	10.4	[1; 22.2]
pH	7.7 (0.7)	8.0	[4.7; 8.4]
CaCO_3 (g.kg^{-1})	84.6 (161.2)	3.3	[0; 835]
Clay (%)	33.3 (9.5)	34.3	[8; 61.7]
Silt (%)	57.9 (9.6)	56.7	[35.5; 86.2]
Sand (%)	8.8 (4.8)	7.4	[2; 29.3]
Microbial characteristics			
Microbial biomass	65.2 (55.9)	48.5	[2.28; 372.0]
Bacterial richness	1276.2 (145.3)	1262.0	[850; 1761.0]
Bacterial evenness	0.8 (0.02)	0.8	[0.7; 0.8]
Bacterial Shannon index	5.5 (0.2)	5.5	[4.5; 6.1]

pesticide treatment frequency index and crop rotation diversity (number of plant types) were not discriminating. These clusters followed a gradient in cropping intensity and in the diversity and persistence of plant cover, that is, Forest (forest, no tillage, no catch crop, $n = 44$); Perennial crop (three frequently mowed grasslands, three blackcurrant and 1 *Miscanthus*, $n = 7$); Catch crop (agricultural plot, minimum tillage, catch crop, $n = 22$); Minimum tillage (agricultural plot, minimum tillage, no catch crop, $n = 57$); Conventional tillage (agricultural plot, conventional tillage, no catch crop, $n = 104$); Mechanical hoeing (agricultural plot, mechanical hoeing, no catch crop, $n = 33$).

Agricultural plots in the conventional tillage and mechanical hoeing clusters were mainly situated between the villages of “Chevigny” and “Fénay” whereas most plots in the minimum tillage cluster (with or without catch crop) were found to the extreme south west and south east. The forests plots were mainly situated beside the two rivers (“La Sans Fond” and “Grand Fossé”, Fig. 1F).

Landscape distribution of molecular microbial biomass

The amount of DNA recovered from the 278 soils of the landscape ranged from 2.28 to 372.0 $\mu\text{g DNA.g}^{-1}$ dry soil (Table 1). The mean recovery was 65.2 $\mu\text{g DNA.g}^{-1}$ dry soil with most soils (90%) yielding concentrations below 126 $\mu\text{g DNA.g}^{-1}$ dry soil. The map of microbial biomass highlighted its heterogeneous distribution and revealed high values under forest and under agricultural plots close to the “Grand Fossé” river, at the west of “Chevigny” and “Saulon-La-Rue” and at the extreme east of the Fénay landscape (Fig. 2A). The validity of the spatial prediction is confirmed by the cross-validation results (Table S2).

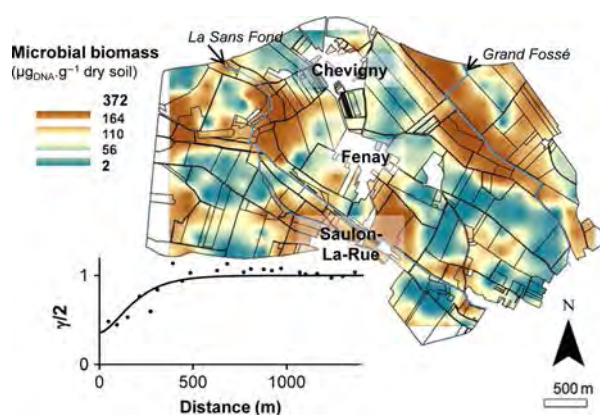


Figure 2. Map and variogram of soil molecular microbial biomass observed at the scale of the Fénay landscape. The color indicates the extrapolated values expressed as μg of DNA.g^{-1} of soil sample. Points represent the experimental variogram, and continuous lines the Matérn models fitted by maximum likelihood method. Geostatistics and cross-validation parameters are provided in Table S2.

The fitted model gave an effective range of 521 m (Fig. 2, Table S2) confirming the spatial structure of microbial biomass in patches of several hundreds of meters across the Fénay landscape. Moreover, the small value of the ν parameter indicated a rough spatial process over small distances (Table S2).

Landscape distribution of bacterial diversity

Pyrosequencing of 16S rRNA genes yielded a total of 5.10^6 sequences (10,800 quality sequences per sample). The rarefaction curves of bacterial OTU confirmed that our sequencing effort allowed a fine description of the bacterial diversity in each soil sample (data not shown). Bacterial richness across the Fénay landscape ranged from 850 to 1,761 OTU with a mean of 1,276 OTU (Table 1). Most soils (85%) exhibited a bacterial richness between 1,100 and 1,480 OTU (Table 1). Soil bacterial evenness ranged from 0.64 to 0.83 with a mean of 0.77 and most samples (90%) exhibited an evenness value >0.74 . Shannon index ranged from 4.43 to 6.17 with a mean of 5.2. Eighty percent of the soils gave values between 5.3 and 5.9 (Table 1).

Visual examination of maps of bacterial richness, evenness, and Shannon index evidenced a heterogeneous distribution and broad similar patterns (Fig. 3A–C). However, a more precise inspection revealed several differences between bacterial diversity parameters with hotspots of bacterial richness located all along the “Sans Fond” river as well as at the east of “Chevigny” and “Saulon-La-Rue” villages (Fig. 3A). Bacterial evenness was distributed in more numerous and smaller patches than bacterial richness, with high values located between the “Fénay” and “Saulon-La-Rue” villages and cold spots in

the north east of “Chevigny” (Fig. 3B). The interpolated map of Shannon diversity index showed an intermediate distribution between bacterial richness and evenness with hotspots of diversity along the “Sans Fond” river as well as at the east of “Saulon-La-Rue”, whereas cold spots were found in the north east of the landscape (Fig. 3C).

The results of the cross-validation confirmed the validity of the spatial predictions of the bacterial diversity

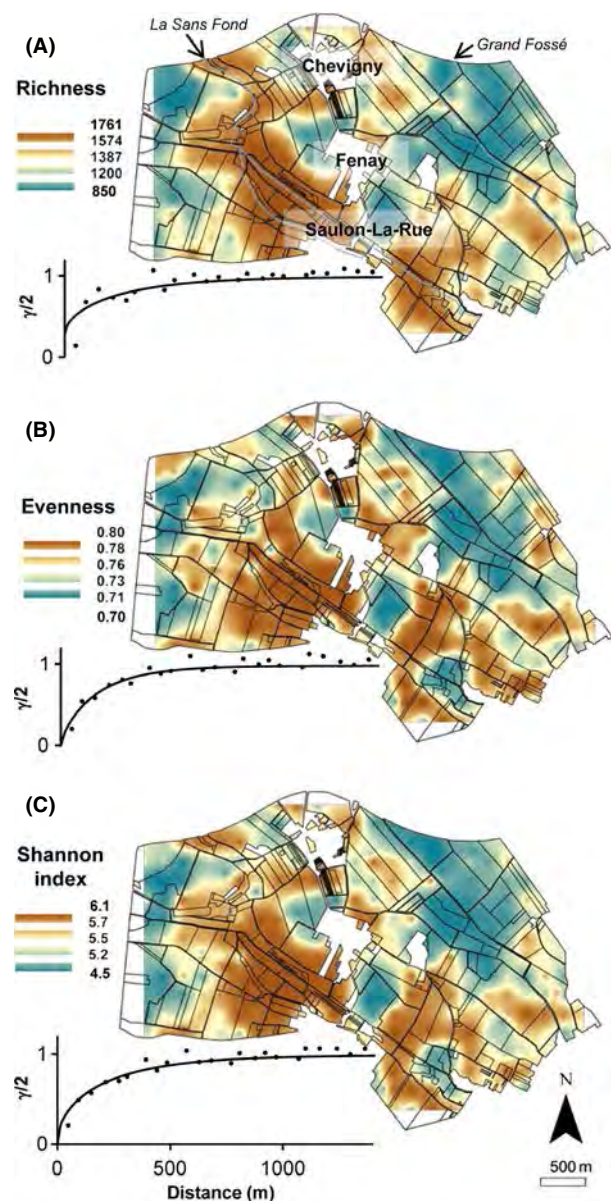


Figure 3. Maps of (A) bacterial richness, (B) bacterial evenness, and (C) bacterial Shannon index parameters measured on the scale of the Fenay landscape. The color indicates the extrapolated values. Points represent the experimental variogram, and continuous lines the Matérn models fitted by maximum likelihood method. Geostatistics and cross-validation parameters are provided in Table S2.

(Table S2). The fitted Matérn models showed effective ranges of 807 m, 521 m, and 758 m for bacterial richness, bacterial evenness, and Shannon index, respectively (Fig. 3, Table S2). The small values of ν parameter indicated rough spatial processes of bacterial diversity over a small distance (Table S2).

Variance partitioning of microbial community

The partial regression models demonstrated a systematically significant influence of soil characteristics, land management, and spatial descriptors on microbial biomass and bacterial diversity variation. The total amount of explained variance was 78.1% for microbial biomass, and 54.6%, 74.4%, and 73.1% for bacterial richness, evenness, and Shannon index, respectively (Fig. 4). Soil characteristics were the best predictors of microbial biomass (21.4%), bacterial richness (43.7%), and Shannon diversity index (29.3%) whereas land management was the best descriptor of bacterial evenness (32.4%, Fig. 4) which was not explained by the spatial variations of the environmental variables. Physicochemical parameters and land management clusters jointly explained a large amount of the total variance (from 4.8% to 34.2%, Fig. 4) that could not be tested.

The marginal effects of each filter within the sets of soil characteristics and spatial descriptors were ranked according to their respective amounts of variance explained, and to their standardized estimated coefficients (Table 2). For each filter, the marginal effect accounted for relatively

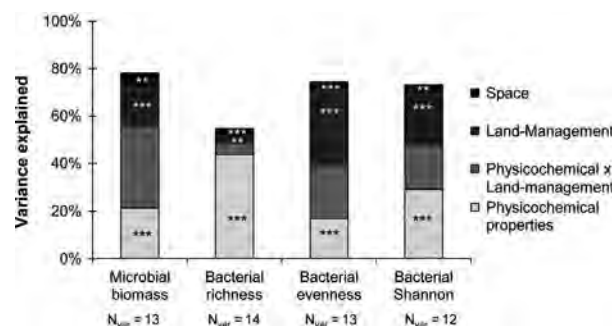


Figure 4. Variance partitioning of molecular microbial biomass and bacterial diversity parameters. The amount of explained variance corresponds to the adjusted r^2 values of the contextual groups using partial redundancy analysis: □ soil physicochemical characteristics; ■ land management ■ space; ■ shared amount of variance between soil characteristics and land management that could not be tested. The significance level of the contribution of the sets of variables is indicated as follows ** $P < 0.01$ and *** $P < 0.001$. N_{var} is the number of explanatory variables retained after selecting the most parsimonious explanatory variables (by minimizing the AIC, akaike information criterion).

small, but significant, proportions of the total variance (from 0.1% to 10%) due to the large number of parameters involved. Regarding the soil characteristics, organic carbon content (10.1%), C:N ratio (1.5%) and clay content (0.5%) were the main drivers of microbial biomass, with organic carbon and clay content having a positive effect (indicated by a positive sign for the standardized coefficient) and C:N ratio a negative effect (Table 2). The positive influence of soil organic carbon might be partly explained by the fact that microbial biomass represents a proportion (between 2% and 5%) of soil organic matter. On the other hand, pH, clay, and CaCO₃ contents were the main drivers of bacterial richness, evenness, and Shannon index (explained variance ranging from 0.8% to 6.1%) with pH and CaCO₃ having a positive influence and clay content a negative influence (Table 2).

Land management was not included in the filter ranking since it was impossible to determine the relative contributions of each category. However, comparison of the signs and values of the standardized estimated coefficients highlighted a contrasting influence of forest and perennial crops vs. annual crops (Table 2). More precisely, increase of the microbial biomass by land management categories followed the sequence: Forest>Perennial crops>Catch Crop≈Conventional tillage>Mechanical Hoeing>Minimum Tillage. An opposite trend was highlighted for bacterial diversity parameters with annual croplands having a positive influence and forest and perennial crops having a negative one (Table 2), following the sequence: Mechanical Hoeing>Catch Crop>Conventional Tillage>Minimum Tillage>Perennial Crops>Forest (Table 2).

The spatial descriptors of the studied area corresponded to 76 significant PCNM vectors, each representing different spatial scales (coarse, medium, and fine, Table 2). The variance explained by spatial descriptors, independently of environmental variables, ranged from 0.3% to 1.6% of the total variance. Spatial descriptors representing coarse and medium scales were mainly involved in microbial biomass and bacterial diversity distribution. Fine scale descriptors were only involved in bacterial evenness and Shannon index. The influence of the scale was ranked, as described above, by comparing the signs of the standardized coefficients. Both positive and negative influences of spatial descriptors were highlighted to explain variations in microbial biomass whereas only negative influences were highlighted for bacterial diversity. Therefore, microbial biomass distribution was mainly explained by coarse (PCNM₁₁, 804 m radius) and medium scales (PCNM₂₅ and PCNM₁₅, 670 and 630 m radius), the coarse scale having a positive influence and the medium scale a negative one. A negative effect on bacterial richness was also highlighted at the scales of PCNM₁₁ (804 m radius) and PCNM₂₄ (624 m radius). A

larger number of PCNMs were involved in explaining bacterial evenness and Shannon variations, describing coarse scale (PCNM₃ and PCNM₂, for bacterial evenness and Shannon, respectively), medium scale (PCNM₂₁ for both evenness and Shannon), and fine scale (PCNM₅₉ and PCNM₄₄, respectively).

Discussion

Most recent studies of soil microbial biogeography have highlighted the major contribution of proximal soil characteristics as drivers of microbial community (Fierer and Jackson 2006; Griffiths *et al.* 2011). However, the considerable soil heterogeneity occurring on a wide scale may mask other drivers associated with human activities, such as agricultural or industrial practices (Fierer and Ladau 2012). Here, we studied microbial distribution across a landscape, which represents the scale of human activities, to better identify and rank environmental versus land management drivers.

The landscape studied was mainly characterized by alkaline silty soils and a mosaic of different types of land management constituted by forest (18% of the area) and agricultural plots with contrasting agricultural practices (82% of the area). The soil characteristics were spatially structured in patches ranging from 600 m to 800 m, which matched the variations in pedological patterns (data not shown) and the distribution of land management categories (Fig. 1). Indeed, lower pH and higher organic carbon, nitrogen contents and C:N ratio were recorded under forest as classically observed (Arrouays *et al.* 2001). Soil characteristics also matched with landscape geomorphology and especially with the “Sans fond” river. Regarding land management, the forest plots were located along the two rivers whereas the agricultural plots distribution did not match with either landscape geomorphology or pedological patterns.

The amount of soil DNA recovered from the 278 soils under study was within the range classically obtained in soil environments with various soil protocols (Plassart *et al.* 2012). The great range of variations recorded across the landscape was similar to that observed on the French territory scale (Dequiedt *et al.* 2011), thus supporting the considerable variability of microbial biomass at both local and global scales. Geostatistical predictions of DNA recovery provided the first map of microbial biomass at this scale. As indicated by the variogram model parameters, the heterogeneous distribution of microbial biomass showed significant spatial organization into patches of several hundreds of meters (about 521 m in radius, Fig. 2). A similar heterogeneous and spatially structured distribution was observed at both smaller and larger scales with patches ranging from several millimeters at the soil microscale (Nunan *et al.* 2003), several tens of meters at the plot scale

Table 2. Model parameters for the distribution of microbial biomass and bacterial diversity within the Féray Landscape.

	Microbial biomass		Bacterial richness		Bacterial evenness		Shannon index	
	Model coefficient	Explained variance (%)	Model coefficient	Explained variance (%)	Model coefficient	Explained variance (%)	Model coefficient	Explained variance (%)
Physicochemical								
Organic carbon	0.035	10.1***	0.012	0.3*				
C:N ratio	-0.010	1.5***						
pH			0.027	5.9***	0.021	5.7***	0.025	7.3***
CaCO3	-0.005	0.5*	0.019	2.1**	0.008	0.6*	0.009	1.7**
Clay	0.007	0.5*	-0.022	6.1***	-0.010	1.5***	-0.008	2.5**
Sand			0.010	0.8*	0.004	0.3 ^{ns}	0.005	0.6*
Land management								
Forest	0.076		-0.029		-0.111		-0.086	
Perennial crop	0.012		-0.037		-0.006		-0.007	
Catch crop	-0.002		0.034		0.008		0.019	
Min. tillage	-0.005	17.3***	0.022	3.7***	0.002	32.4***	0.010	23.1***
Mech. hoeing	-0.004		0.035		0.029		0.033	
Conv. hillage	-0.002		0.026		0.017		0.023	
Space								
Coarse Scale [800; 1280]m								
PCNM ₁								
PCNM ₂					-0.006	0.6*		0.6*
PCNM ₃					-0.007	1.1***		
PCNM ₆					-0.005	0.4*		
PCNM ₁₁	0.006	0.8***	-0.008	1.0*				
PCNM ₁₃								
PCNM ₁₅	-0.005	0.6**						
Medium scale [631;800]m								
PCNM ₂₁								
PCNM ₂₄								
PCNM ₂₅	-0.006	0.7**	-0.011	1.6**	-0.006	0.8**	-0.006	0.7**
PCNM ₂₆	0.005	0.4*						
PCNM ₃₂	0.004	0.3*						
Fine scale [445; 631]m								
PCNM ₄₄								
PCNM ₄₇	-0.004	0.3 ^{ns}						
PCNM ₅₉					-0.005	0.5**		0.6*

Each parameter is presented with its estimated model coefficients and its marginal effect assessed by a permutation test. ns, not significant. Missing values indicate that the variable was not retained in the model. Silt was removed prior to model evaluation since it was represented by the opposite of the sum of silt and clay contents. Spatial components were summarized according to the spatial scale considered: coarse, medium or fine.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Berner *et al.* 2011; several hundreds of kilometers at the territory scale (Dequiedt *et al.* 2011), to several thousands of kilometers at the earth scale (Serna-Chavez *et al.* 2013).

Visual comparison of maps of microbial biomass and environmental characteristics suggested that microbial abundance was influenced by both land management and soil characteristics. Microbial biomass hot spots matched with forest plots, and cold spots with croplands, which also corresponded to the distributions of soil organic carbon contents and C:N ratio. Variance partitioning of microbial biomass revealed that soil characteristics were the main drivers, as previously reported on a larger scale (Dequiedt *et al.* 2011; Serna-Chavez *et al.* 2013). More precisely, organic carbon content and C:N ratio were the primary drivers influencing microbial biomass with a positive and negative effects, respectively. This is consistent with several reports that organic carbon availability and soil organic matter recalcitrance to degradation by microbes are related to the abundance of microorganisms (Leckie *et al.* 2004; de Boer *et al.* 2005). However, a weak influence of clay content was also recorded, which is not consistent with environmental filters hierarchy observed on a broader scale (Dequiedt *et al.* 2011). This difference might partly be explained by the smaller variation in soil texture measured on our landscape scale, as compared to the French territory scale (Coefficient Variation [CV] = 16.5% vs. 43.6%; respectively) and contrary to the variations in quantity and quality of organic carbon (CV = 72.1% vs. 80.0%, and 37.1% vs. 15.0%, respectively, Ranjard *et al.* 2013).

Analysis of the marginal effect of land management categories revealed a negative impact of croplands on microbial biomass but not of forests (Table 2). This could be due to the high organic matter content of soil under forest management as compared to the low organic carbon content observed in soils under conventional crops (Arrouays *et al.* 2001). Comparison of the types of agricultural managements revealed differences only between perennial and non perennial crops, thus, confirming the stimulation of microbial abundance under permanent and diversified plant cover (Lienhard *et al.* 2014). However, no difference in the effects of tillage regime were observed, which contrasts with recurrent reports of a significant loss of microbial biomass with increased soil disturbance (Govaerts *et al.* 2007; Lienhard *et al.* 2014). This discrepancy could result from the covariation of tillage regimes with certain soil characteristics in our landscape (e.g., soil organic carbon and texture), which might have increased the amount of variance explained by interaction between land management and soil characteristics, and hampered our evaluation of the impact of particular agricultural practices.

Characterization of bacterial diversity by pyrosequencing of 16S rDNA from soil DNA revealed significant spa-

tial variations in bacterial richness, evenness, and Shannon index across the landscape which were in agreement with other studies covering variations in physico-chemical and land management characteristics at similar or broader spatial scales (Nacke *et al.* 2011; Shange *et al.* 2012). Geostatistical interpolation showed spatial patterns characterized by patches of 807 m (richness), 521 m (evenness), and 758 m (Shannon index). The maps of bacterial richness and microbial biomass did not match, confirming that microbial abundance and diversity can be influenced by different drivers (Fierer and Jackson 2006; Dequiedt *et al.* 2009, 2011). These different patterns might be partially related to the contribution of fungi, protozoa, and other eukaryotes to the DNA pool, which may be under the dependence of drivers different from those of bacterial biomass. Hot spots of richness seemed to occur in the vicinity of the “*Sans fond*” river, suggesting a strong influence of landscape geomorphology but also of soil characteristics since the soils all along this river were alkaline with high soil organic carbon and sand contents. Spatial distributions of bacterial evenness and Shannon index were fairly similar to richness but smaller patches were also apparent, suggesting an impact of other environmental filters. Variance partitioning confirmed the different determinisms of richness and evenness, with richness being mainly influenced by soil characteristics and evenness by land management. This is congruent with recent studies evidencing the major effect of soil characteristics on bacterial richness (Lauber *et al.* 2009; Kuramae *et al.* 2012; Rodrigues *et al.* 2013). Our results support that soil characteristics influence the number of species by modulating soil habitat heterogeneity whereas land management mostly influences bacterial population equilibrium by modulating environmental perturbation.

Focusing more precisely on soil characteristics, our study emphasized the overriding effect of pH as a stimulating factor of bacterial community diversity (richness, evenness, and therefore Shannon index) at various spatial scales (Fierer and Jackson 2006; Green and Bohannan 2006; Rousk *et al.* 2010). Clay content also appeared to be a significant driver of bacterial richness, evenness, and Shannon index variation but had a deleterious effect. Thus, fine textured soil harbored a large microbial biomass, due to its more extensive microhabitats leading to a high carrying capacity, but only a small number of bacterial species, due partly to the reduced heterogeneity leading to a lesser diversity of microbial habitats at the soil microscale (Carson *et al.* 2010; Chau *et al.* 2011). In addition, the reduced evenness might result from the increase of competitive exclusion between populations due to the high homogeneity of soil microhabitats. This observation might be also partly explained by the high level of protection provided by fine texture soil for the

bacterial community against environmental perturbations (Chenu *et al.* 2001; Constancias *et al.* 2013), leading to a decreased population equilibrium through a diminution of selection process between populations (Giller *et al.* 1998; Bressan *et al.* 2008).

Independently of other environmental variables, land management accounted for a small proportion (3.7%) of the explained variance for bacterial richness, in agreement with previous reports that bacterial richness is generally poorly impacted by land use (Enwall *et al.* 2010; Kuramae *et al.* 2012). Interestingly, bacterial richness was lower in forest soils (mean of 1191 OTUs) than in crop soils (mean of 1297 OTUs), whereas microbial biomass was strongly stimulated under forest (159 $\mu\text{g DNA.g}^{-1}$ soil for forest soils vs. 47 $\mu\text{g DNA.g}^{-1}$ soil for crop soils). A similar and more significant trend was observed in the positive effect of crop soils on evenness and Shannon index (0.73 vs. 0.78 for forest and crop soils evenness, respectively; 5.16 vs. 5.58 for forest and crop soils Shannon index, respectively). These diversity parameters were positively related, in crop soils, to the gradient of increased soil disturbance by tillage. This stimulatory effect of tillage on soil bacterial diversity may be related to the degree of perturbation induced by this agricultural practice (Acosta-Martínez *et al.* 2010; Lienhard *et al.* 2014). According to the “hump back” model between biodiversity and the intensity of environmental perturbation, which suggests that the greatest biodiversity is obtained with moderate environmental perturbation due to a diminution in competitive niche exclusion and selection mechanisms occurring between populations (Giller *et al.* 1998), our results emphasize that crop soils under conventional tillage and mechanical hoeing would correspond to these conditions (Lienhard *et al.* 2014).

Spatial descriptors, illustrating neighborhood relationships between samples, systematically accounted for the smallest significant contribution to microbial biomass and diversity distributions at coarse (800–1280 m), medium (630–800 m), and fine scales (440–630 m). In agreement with Hanson *et al.* (2012), the influence of spatial descriptors might be partly related to variations in unmeasured soil characteristics at the medium scale, whereas it might result from landscape configuration at the coarse and fine scales. The coarse scale represents the global distribution of forest vs. crop patches, and the fine scale represents the distribution of individual agricultural plots subjected to particular practices. These results suggest that landscape configuration would be an additional driver of soil microbial biomass and bacterial diversity distribution. This hypothesis is in agreement with Ranjard *et al.* (2013), who demonstrated the influence of territory heterogeneity and configuration in shaping bacterial diversity turnover. In addition, our analysis revealed a systematically negative effect of spatial descriptors on bacterial diversity, which

suggests that landscape configuration might partially affect bacterial diversity by limiting bacterial dispersal. This result supports the hypothesis that the selection and dispersal limitation of microbial populations are not exclusive as suggested by Hanson and Fuhrman (2012).

Altogether, our study provides the first map of microbial biomass and bacterial diversity across an agricultural landscape, and demonstrated the heterogeneous but spatially structured distribution of the microbial community at this scale, mainly driven by proximal filters such as soil characteristics and agricultural practices. Our results therefore confirm that the landscape is an appropriate scale for robust evaluation of the influence of agricultural land management on soil microorganisms. This spatial scale is also shown to be relevant for modifying and improving human activities in the context of a sustainable use of soil resources. Further analyses are now required to measure and link soil microbial activities with microbial diversity and to identify and better define the bacterial groups and their ecological attributes at this scale.

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Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Physicochemical characteristics measured at each sampling point.

Figure S2. Microbial community characteristics at each sampling point.

Table S1. Steps, parameters and database used in the bioinformatics analysis of 16s rRNA sequences obtained by pyrosequencing.

Table S2. Geostatistics and cross-validation parameters for the physicochemical and microbial data standardized by Gaussian quantile transformation.

ORIGINAL ARTICLE

Improving soil bacterial taxa–area relationships assessment using DNA meta-barcoding

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The evaluation of the taxa–area relationship (TAR) with molecular fingerprinting data demonstrated the spatial structuration of soil microorganisms and provided insights into the processes shaping their diversity. The increasing use of massive sequencing technologies in biodiversity investigations has now raised the question of the advantages of such technologies over the fingerprinting approach for elucidation of the determinism of soil microbial community assembly in broad-scale biogeographic studies. Our objectives in this study were to compare DNA fingerprinting and meta-barcoding approaches for evaluating soil bacterial TAR and the determinism of soil bacterial community assembly on a broad scale. This comparison was performed on 392 soil samples from four French geographic regions with different levels of environmental heterogeneity. Both molecular approaches demonstrated a TAR with a significant slope but, because of its more sensitive description of soil bacterial community richness, meta-barcoding provided significantly higher and more accurate estimates of turnover rates. Both approaches were useful in evidencing the processes shaping bacterial diversity variations on a broad scale. When different taxonomic resolutions were considered for meta-barcoding data, they significantly influenced the estimation of turnover rates but not the relative importance of each component process. Altogether, DNA meta-barcoding provides a more accurate evaluation of the TAR and may lead to re-examination of the processes shaping soil bacterial community assembly. This should provide new insights into soil microbial ecology in the context of sustainable use of soil resources.

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INTRODUCTION

Soils are highly complex ecosystems and are considered as one of the Earth's main reservoirs of biological diversity. Bacteria account for a major part of this biodiversity, and it is now clear that such microorganisms have a key role in soil functioning processes (for example, control of nutrient cycles, and directly influence plant, animal or human health; Nemergut *et al.*, 2011). However, many of the environmental factors regulating the diversity of below-ground bacteria, still need to be investigated, which limits our understanding of the distribution of such bacteria at various spatial scales (Hanson *et al.*, 2012).

Until recently, most biogeographic studies have been devoted to plants and animals, providing insights into the ecological processes (dispersal, selection, ecological drift and speciation), which shape the community assembly and dynamics of macroorganisms (Nemergut *et al.*, 2011, 2013; Hanson *et al.*, 2012). For microorganisms, the first biogeographic hypothesis was developed by Baas Becking in 1934: 'Everything is everywhere, *but*, the environment selects', implying that microbes would be homogeneously distributed on a broad scale and among various environments. Interestingly, the number of microbial biogeography studies has increased exponentially over the last decade because of progress with molecular tools for routine application and broad-scale sampling networks involving several hundreds of samples

(Maron *et al.*, 2011; Hanson *et al.*, 2012). These studies are providing overwhelming evidence that microorganisms display biogeographic patterns, but that much remains to be described and understood about the ecological processes contributing to these biological distributions as well as their relative importance (Hanson *et al.*, 2012; Ranjard *et al.*, 2013).

The oldest and most relevant way to discriminate the spatial processing of microbial diversification is to evaluate the taxa–area relationship (TAR). The first TAR was reported by Arrhenius (1921) as a power–law relationship between species richness (S_A) in an area A and local species richness (S_0) and area (A):

$$S_A = S_0 A^z \quad (1)$$

In this equation, z represents the rate at which new species are sampled as the sampling area is increased. This has been extended to microorganisms by taking equation (1) and deriving a similarity distance–decay relationship between community similarity between sites and geographic distance between sites (equation 2):

$$\chi_d = \chi_D \left(\frac{d}{D} \right)^{-2z} \quad (2)$$

In this equation, z is the same parameter as in equation (1) and is commonly considered as a turnover rate. χ_d and χ_D are the

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community similarities between sites located d meters and D meters apart from each other. This derivation is based on two assumptions: that community size is infinite and that z is steady (Rosindell *et al.*, 2011). For microorganisms, the infinite community size hypothesis may hold because soil microbial communities are commonly assumed to be very large and diverse, and the average abundance per microbial taxa is high (Harte *et al.*, 2009). On the contrary, the hypothesis of z remaining steady across scales, which assumes self-similarity as a probability rule for the spatial distribution of taxa abundance across spatial scales (Harte *et al.*, 1999), may not hold for soil microbes. Consequently: (i) it may be assumed that the similarity distance–decay relationship is equivalent to the TAR and (ii) the z estimates and subsequent conclusions may vary across scales but can be assumed constant for a given scale.

Nevertheless, beyond the debate concerning the form of the TAR equation, this relationship is assumed to result mainly from: (i) the accumulation of species as the sampling area is increased because of the increased number of different habitats sampled (corresponding to the selection process); (ii) population dynamics, with greater possibilities for colonization and speciation but lower extinction rates in larger areas, corresponding to dispersal limitations and ecological drift processes; and (iii) speciation processes within the considered organisms (Hubbell, 2001; Zinger *et al.*, 2014). Challenging the widespread idea that microorganisms exhibit a cosmopolitan distribution, TAR is now commonly used in a majority of microbial biogeographical studies to assess microbial community turnover rate and its relative potential dependence on ‘dispersal’ and ‘selection’ (Angel *et al.*, 2010; Martiny *et al.*, 2011; Ranjard *et al.*, 2013; Wang *et al.*, 2013; Zinger *et al.*, 2014). The estimated turnover rates for microbial communities in most studies range from 0.002 to 0.26 (Horner-Devine *et al.*, 2004; Green and Bohannan, 2006; Woodcock *et al.*, 2006), and are generally much lower than those estimated for macroorganisms (classical range: 0.1–0.25; Horner-Devine *et al.*, 2004). In addition, Ranjard *et al.* (2013) have shown that selection and limited dispersal are not mutually exclusive and that a non-negligible proportion of bacterial community variation on a broad scale might be explained by the latter.

Although these studies demonstrated a significant spatial structuring of bacterial communities into biogeographical patterns, they were mainly based on molecular approaches with limited resolution, such as fingerprinting methods (Angel *et al.*, 2010; Ranjard *et al.*, 2013), or low-depth sequencing (Martiny *et al.*, 2011). Nowadays, high-throughput sequencing technologies (for example, 454 pyrosequencing or Illumina) are readily available to assess microbial diversity with greater precision and provide huge amounts of taxonomic information, based on hundreds of thousands of ribosomal RNA (rRNA) gene sequences (here designated DNA meta-barcoding) from a single metagenomic DNA (Maron *et al.*, 2011). Increasing use of these technologies in biodiversity investigations raised methodological and conceptual insights to ecologists (Wang *et al.*, 2013; Zinger *et al.*, 2014). Regarding biogeography studies, it has raised the question of the potential gain offered by the greater resolution of the DNA meta-barcoding approach, as compared with fingerprinting, in providing a deeper understanding of the determinism of microbial community assembly on a broad scale (Terrat *et al.*, 2012; Lienhard *et al.*, 2013). Recently, Van Dorst *et al.* (2014) incorporated various spatial scales and demonstrated the similar capacities of DNA fingerprinting and meta-barcoding to discriminate bacterial communities and to correlate with environmental variables at a local scale, but the greater resolution of DNA meta-barcoding at a global scale. This underlines the importance of adopting DNA meta-barcoding to support studies on

broad to global scales, and to reexamine the processes involved in community assembly.

Our objectives in this study were to compare soil bacterial TAR and the determinism of soil bacterial community assembly on a broad scale using both approaches, namely DNA fingerprinting (Automated RISA fingerprinting, ARISA data set in the following) and DNA meta-barcoding (454 pyrosequencing, NGS data set in the following), to characterize soil bacterial diversity. Four geographic regions in the RMQS data set (‘Réseau de Mesures de la Qualité des Sols’ = French Monitoring Network for Soil Quality, covering 2200 soils over the whole of France using a systematic grid 16 km × 16 km) were selected along a gradient of environmental heterogeneity, representing a total of 392 soils. As the meta-barcoding approach can provide taxonomic information at different resolutions, multiple operational taxonomic unit (OTU) clustering thresholds (80 to 97%) were used. The similarity between two communities was determined with the Sørensen index based on the amount of shared OTUs, irrespective of their relationship (Green *et al.*, 2004). In each region, the soil bacterial community turnover rates (z) were estimated using the above-described similarity distance–decay relationship (equation 2). A distance-based redundancy analysis was used to partition bacterial community variance according to pedo-climatic characteristics, land-use and spatial variables. Our main hypothesis was that DNA meta-barcoding would provide a more robust estimation of TAR and a better understanding of the processes involved in bacterial community assembly on a broad scale than molecular fingerprinting.

MATERIALS AND METHODS

Sampling design

Soil samples were provided by the soil genetic resource conservatory of the GenoSol platform (http://www2.dijon.inra.fr/plateforme_genosol/) and obtained from the soil storage facility of the RMQS. The RMQS database consists of soil samples obtained from a regular 16-km grid across the 550 000 km² of metropolitan France and was designed to monitor soil properties (Arrouays *et al.*, 2002). The baseline survey comprises 2200 sites (each corresponding to a composite soil sample obtained from 25 soil cores) and was started in 2001 and completed in 2009. No temporal effect has been observed (data not shown). The 392 sites analyzed in this study were organized into four geographic regions: Brittany (124 sites), Burgundy (109 sites), Landes (52 sites) and South-East (107 sites) with contrasting soil type, land-use (coarse level of the CORINE Land Cover classification; IFEN, <http://www.ifen.fr>; 7 classes: forest, crop systems, grasslands, particular natural ecosystems, vineyards/orchards, parkland and wild land), climate (Quintana-Segui *et al.*, 2008) and geomorphology (Supplementary Table S1). The sites within a region were at least 16-km apart. For each soil, the following pedo-climatic characteristics were examined: particle-size distribution, pH in water (pH_{water}), C:N ratio, organic carbon (C_{org}), N, soluble P, CaCO₃ and exchangeable cation (Ca, K and Mg) contents, sum of annual temperatures (°C) and annual rainfall (mm). Physical and chemical analyses were performed by the Soil Analysis Laboratory of INRA (Arras, France), which is accredited for such analyses by the French Ministry of Agriculture.

DNA molecular fingerprinting data (fingerprint data set)

The subset of 392 soil samples was selected from the DNA fingerprinting data and methods (DNA extraction, purification, quantification and automated ribosomal intergenic spacer analysis), originally described and analyzed in the study by Ranjard *et al.* (2013). After automated ribosomal intergenic spacer analysis, contingency tables were derived from the fingerprints with samples in lines and bands (referred to as OTU_{bin} in the following) in columns with a maximum of 100 bands per sample to avoid taking into account artefactual bands because of image analysis.

DNA meta-barcoding data (NGS data set)

Soil DNA extraction, purification and quantification. Microbial DNA was freshly extracted from soils using a procedure optimized by the GenoSol platform named GnS-GII (Plassart *et al.*, 2012; Terrat *et al.*, 2012). The main difference between the GnS-GII and the DNA extraction procedure used in Ranjard *et al.* (2013) is the grinding step. However, both of these DNA extraction methods can provide a representative picture of the community with DNA molecular fingerprinting approaches (Plassart *et al.*, 2012), but not if high-throughput sequencing technologies, with their greater resolution, are used (Terrat *et al.*, 2012). μl Aliquots of crude DNA extracts were loaded onto polyvinylpyrrolidone microbiospin minicolumns (BIO-RAD Laboratories, Marnes-la-Coquette, France) and centrifuged for 4 min at 1000 *g* and 10 °C. Eluates were then collected and purified for residual impurities using the GeneClean Turbo kit (MP-Biomedicals, New-York, NY, USA). Purified DNA extracts were then quantified using the PicoGreen staining Kit (Molecular Probes, Paris, France).

Pyrosequencing of 16S rRNA genes. Microbial diversity (*bacteria* and *archaea*) was determined for each soil by 454 pyrosequencing of ribosomal genes. A 16S rRNA gene fragment targeting the complete hypervariable regions V4 (576–682) and V5 (822–879) (numbering based on the *Escherichia coli* system of nomenclature (Brosius *et al.*, 1978)) with an appropriate size (about 450 bases) for 454-pyrosequencing was amplified using the primers F479 (5'-CAGC MGCYGCNGTAANAC-3') and R888 (5'-CCGYCAATTCMTTTRAGT-3'). Homemade bioinformatic programs have been developed to search large DNA sequence databases for the presence of primers, including degeneracies, as coded by the IUPAC rules, and also additional mismatches in order to test primer improvement. The sequences investigated were SILVA, and direct extraction of every small subunit rRNA sequence from EMBL using acnuc, and also a dedicated reference database of 18S eukaryotic sequences, which had been thoroughly analyzed and annotated (Supplementary Table S2) for *in silico* match analysis. For each sample, 5 ng of DNA were used for a 25 μl PCR conducted under the following conditions: 94 °C for 2 min, 35 cycles of 30 s at 94 °C (denaturation), 30 s at 52 °C (hybridization) and 1 min at 72 °C (elongation), followed by 7 min at 72 °C (final elongation). The PCR products were purified using a MinElute gel extraction kit (Qiagen, Courtaboeuf, France) and quantified using the PicoGreen staining Kit (Molecular Probes). A second PCR of nine cycles was then conducted under similar PCR conditions with 5 ng of purified PCR products and 10-base pair multiplex identifiers, designed and validated by ROCHE (http://www.liv.ac.uk/media/livacuk/centreforgenomicresearch/The_GS_FLX_Titanium_Chemistry_Extended_MID_Set.pdf) and added before the 5' position of the primers, and after the 3' positions of the adapters to specifically identify each sample and avoid PCR bias. Finally, the PCR products were purified and quantified as described above. Pyrosequencing was then carried out on a GS FLX Titanium (Roche 454 Sequencing System) at Genoscope (Evry, France).

Bioinformatic analysis of 16S rRNA gene sequences. Bioinformatic analyses were done using the GnS-PIPE initially developed by the GenoSol platform (INRA, Dijon, France; Terrat *et al.*, 2012), and recently optimized. The parameters chosen for each bioinformatic step can be found in Supplementary Table S3. First, all the 16S raw reads were sorted according to the multiplex identifier sequences. The raw reads were then preprocessed (filtered and deleted) based on: (a) their minimum length, (b) their number of ambiguities (Ns) and (c) their primer sequences. A PERL program was then applied for rigorous de-replication (that is, clustering of strictly identical sequences). The de-replicated reads were then aligned using Infernal alignments, and clustered into OTUs using a PERL program that groups rare reads with abundant ones, and does not count differences in homopolymer lengths (here, a cluster is defined by the most abundant read, known as the centroid, and every read in the cluster must have similarity above the given identity threshold with the centroid). A filtering step was then carried out to check all single-singletons (reads detected only once and not clustered, which might be artifacts, such as PCR chimeras) based on the quality of their taxonomic assignments. More precisely, each single-singleton was compared with a dedicated reference database from the Silva curated database using similarity approaches (USEARCH), with sequences longer than 500 nucleotides, and kept only if their identity was higher than the

defined threshold (Supplementary Table S3). When several reference sequences were found (defined maximum of 10), a taxonomic consensus was derived, that is, a read was assigned to a given taxonomy only if a majority of similar reference sequences had the same description. Finally, in order to compare the data sets efficiently and avoid biased community comparisons, a homogenization step of kept reads per sample was carried out, to a value close to the lowest observed among samples (9410 reads), by random selection (Gihring *et al.*, 2012). The global analysis of soil samples was then computed by merging all homogenized high-quality reads from each sample into one global file before subsequent analyses. As the global analysis of bacterial community structure and diversity relies on the construction of similarity clusters (or OTUs) of 16S rRNA gene PCR amplicons (Horner-Devine *et al.*, 2004), we chose to use OTUs to examine the distribution of 16S rRNA gene sequences in our data set. However, there is no single best definition of 'species', 'genus', etc... when this sequencing approach is used, (because of controversy about thresholds of similarity allowing clear differentiation of taxonomic units), so we applied the following thresholds of sequence similarity: 80, 85, 90, 95 and 97% (Rosselló-Mora and Amann, 2001; Nemerget *et al.*, 2011). Such multiple OTU definitions are analogous to comparing different taxonomic resolutions. The retained high-quality reads were then used to determine the OTU composition of samples at each level of similarity. Finally, contingency tables of OTUs were obtained with samples in lines and OTUs in columns, indicating the number of reads in each OTU for all samples. OTUs were also taxonomically assigned using the information from high-quality reads (Supplementary Table S4). The raw data sets are available in the EBI database system under project accession number PRJEB6290.

Data set post-processing. Two filtering steps were applied to the NGS contingency tables of OTUs to eliminate potentially artefactual data (because of sequencing errors or PCR chimeras for example). The first step consisted of removing OTUs that occurred only once in the overall data set, considered as experimental artifacts. The second filtering step was designed to avoid up-weighting the importance of rare OTUs in the data set (as the contingency tables would be converted into binary tables for statistical analyses). This filtering step consisted of changing the value for the sample in the global OTU to 0 if two conditions were verified: (i) for each OTU in the global contingency table, if the reads of one sample represented <1% of the total abundance of the OTU and (ii) if the reads from the given OTU for the analyzed sample represented <0.1% of the total number of cleaned sequences identified in the sample. This second filtering step made it possible to remove less information than a single filter, which removed OTU representing <0.1% of the total number of cleaned sequences identified in the sample (Supplementary Table S5). The contingency tables of OTUs (with samples in lines and OTUs in columns) were then converted into binary tables for subsequent analyses. OTU richness was compared between the raw data set and the filtered data set to evaluate the effects of the filters.

Statistical analyses

Evaluation of congruence between ARISA and 454 sequencing data sets. In order to evaluate if ARISA and 454 sequencing data sets were comparable, the correlation between the distance matrices derived from each data set (Sørensen index) was tested by means of Mantel test (*mantel* function in *vegan* package in R).

Environmental heterogeneity. The level of environmental heterogeneity between regions was determined by applying a multivariate analysis on mixed data using the *ade4* package in R (<http://cran.r-project.org/web/packages/ade4/index.html>) with soil pedo-climatic characteristics, land-use and geomorphological data (elevation). Quantitative data were centered and scaled and qualitative data were converted into weighted binary variables (weight equal to $1/n$; n being the number of classes for the qualitative variables). Differences between the four regions were examined by between-group analysis and a Monte-Carlo permutation test (1000 permutations). The environmental heterogeneity in each region was determined from the site dispersion on the factorial map.

Evaluation of bacterial community composition turnover rate. The turnover rates (z) for bacterial community composition were derived from the slope of the TAR as described in Ranjard *et al.* (2013) following the method described in Harte *et al.* (1999) and by applying equation (3), which is the

log-transformation of equation (2):

$$\log_{10}(\chi_d) = (-2 * z) * \log_{10}(d) + b \quad (3)$$

where χ_d is the observed Sørensen's similarity between two soil samples that are d meters apart from each other; b is the intercept of the linear relationship and z the turnover rate of the community composition. In this study, z is assumed to be constant, that is, independent of d . The z estimate and its 95% confidence interval were derived from the slope ($-2*z$) of the relationship between similarity and distance by weighted linear regression. The overlap of the 95% confidence intervals was used to test for significant differences in turnover rates between regions or methods.

Variance partitioning of community assembly according to environmental filters and space. The relative importance of residual spatial autocorrelation, pedo-climatic characteristics and land-use in determining community composition turnover was tested by db-RDA (Legendre *et al.*, 2005; Legendre and Fortin, 2010). Quantitative data were centered and scaled. Residual spatial autocorrelation was considered by introducing spatial variables into the analysis. These spatial variables were constructed from site coordinates (x , y , elevation) to reveal potential spatial trends at scales larger than the region, and from principal coordinates of neighbour matrices eigenfunctions in each region (pcnm function in *vegan* package, <http://cran.r-project.org/web/packages/vegan/index.html>). Only principal coordinates of neighbour matrices with a significant Moran index ($P < 0.001$) were selected. Land-use corresponded to the CORINE Land Cover classes (IFEN, <http://www.ifen.fr>) recoded into dummy variables. Pedo-climatic characteristics consisted of climate and all the physico-chemical variables except sand. The most parsimonious model was obtained by forward selection from null to full model. The marginal effects of each set of filters were tested with an analysis of variance (ANOVA)-like permutation test for canonical analyses (*anova.cca* function in *vegan* package, <http://cran.r-project.org/web/packages/vegan/index.html>).

RESULTS

Environmental heterogeneity

The four regions were selected for their contrasting environmental heterogeneity as demonstrated by principal component analysis of

mixed data (Figure 1a). This multivariate analysis resulted in the discrimination of the four regions on both axes (Monte-Carlo permutation test, $P < 0.001$). On the first axis, Landes was significantly discriminated from Brittany, Burgundy and South-East, and these three regions were discriminated from each other on the second axis. The environmental heterogeneity differed strongly between regions, the Landes sites being less dispersed on the factorial map than the Brittany or Burgundy sites, which were less dispersed than those of the South-East. Figure 1b shows that the four regions could mainly be distinguished according to land-use (for example, 86% of the Landes sites are forest sites), a restricted set of soil physico-chemical characteristics (sand and silt contents, pHwater and CaCO₃ content, P content and C:N ratio) and by differences in elevation. Climatic conditions did not have a significant role in discrimination between regions.

Post-processing and taxonomic resolution on NGS data

Post-processing steps were applied to the NGS data set in order to account for potentially artefactual data. The effects of the filters were assessed by comparing OTU richness in the raw and filtered data sets. OTU richness at the 80% similarity threshold ranged from 20 to 171 OTUs in the raw data set and from 10 to 126 OTUs in the filtered data set. It increased with the increasing similarity threshold in the clustering analysis to reach a maximum at 97% sequence similarity. At this level of sequence similarity, OTU richness ranged from 106 to 4687 OTUs in the raw data set and from 44 to 1641 OTUs in the filtered data set (Table 1). The filtering steps reduced the number of OTUs considered by 34 to 48%. This reduction was similar for each region and harbored only a slight increase as the similarity threshold increased, ranging from 34 to 45% at the 80% sequence similarity threshold, and from 39 to 48% at the 97% sequence similarity threshold.

Regarding the raw data set, the four regions could be ranked according to the median OTU richness across all sites:

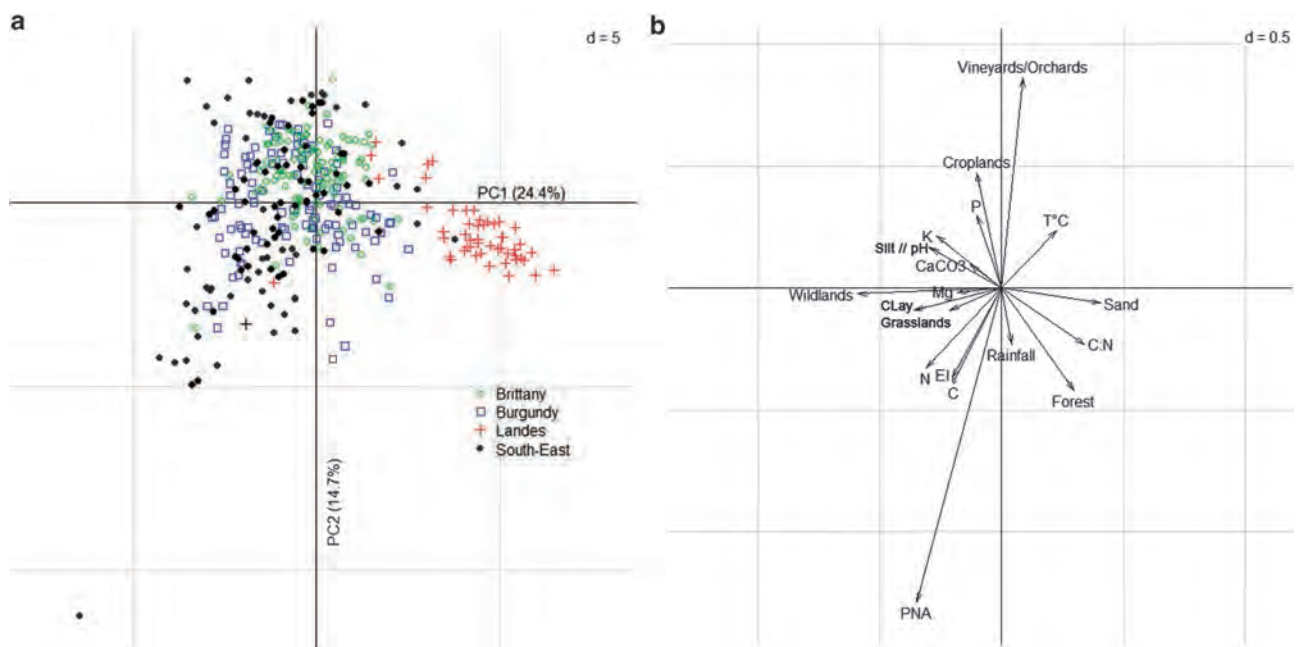


Figure 1 Environmental heterogeneity between the Brittany, Burgundy, Landes and South-East regions. (a) Factorial map representing the sites considered respectively to their region of origin in a principle component analysis on mixed data. Green open opened circles: Brittany; blue open opened squares: Burgundy; red crosses: Landes, black filled circles: South-East. (b) Correlation circle of the principle component analysis on mixed data. Monte-Carlo permutation test (1000 permutations) was significant ($P < 0.001$).

Table 1 OTU numbers in the raw and filtered data sets

Clustering threshold	Region (n)	OTU richness						Removed OTUs (%)
		Raw data set			Filtered data set			
		Min	Median (\pm s.d.)	Max	Min	Median (\pm s.d.)	Max	
80%	Burgundy (109)	36	68 (\pm 16.6)	115	20	39 (\pm 11.5)	70	43
	Brittany (131)	45	92 (\pm 17.9)	152	29	57 (\pm 13.5)	125	38
	Landes (54)	43	60 (\pm 19.3)	120	27	39 (\pm 12.7)	90	34
	South-East (108)	20	86 (\pm 21.0)	171	10	48 (\pm 17.2)	126	45
85%	Burgundy (109)	83	156 (\pm 37.4)	241	44	88 (\pm 25.2)	153	44
	Brittany (131)	105	211 (\pm 39.3)	360	52	123 (\pm 29.9)	274	42
	Landes (54)	94	144 (\pm 61.8)	435	62	90 (\pm 43.8)	321	38
	South-East (108)	33	211 (\pm 42.6)	377	15	120 (\pm 33.6)	263	43
90%	Burgundy (109)	183	344 (\pm 84.9)	577	91	181 (\pm 55.0)	367	47
	Brittany (131)	217	488 (\pm 99.9)	869	125	260 (\pm 62.7)	478	47
	Landes (54)	201	343 (\pm 188.5)	1361	136	223 (\pm 133.8)	935	35
	South-East (108)	56	485 (\pm 95.2)	835	22	277 (\pm 71.6)	483	43
95%	Burgundy (109)	428	841 (\pm 188.8)	1376	245	435 (\pm 110.0)	828	48
	Brittany (131)	473	1171 (\pm 227.0)	1862	316	628 (\pm 127.2)	1106	46
	Landes (54)	475	875 (\pm 496.1)	3423	356	570 (\pm 247.4)	1622	35
	South-East (108)	93	1165 (\pm 209.6)	1638	33	669 (\pm 128.9)	997	43
97%	Burgundy (109)	669	1273 (\pm 267.2)	2061	415	666 (\pm 147.7)	1131	48
	Brittany (131)	758	1752 (\pm 306.4)	2620	494	904 (\pm 143.7)	1392	48
	Landes (54)	712	1360 (\pm 691.1)	4687	541	832 (\pm 249.4)	1641	39
	South-East (108)	106	1725 (\pm 294.4)	2236	44	959 (\pm 163.7)	1295	44

Abbreviation: OTU, operational taxonomic unit.

For the raw and the filtered data sets, the median number of OTUs identified per site, its minimum and its maximum were determined per region at each clustering threshold considered in this study. The s.d. of the mean is given in brackets. The percentage of OTUs removed by filtering steps was estimated by dividing the median number of OTUs in the filtered data set by the median number of OTUs in the raw data set.

Landes \approx Burgundy < Brittany \approx South-East ($P < 0.05$, χ^2 test), whatever the clustering threshold. The same ranking of the different regions was obtained with the filtered data set, indicating that the overall trends in the diversity indices were not affected by the filtering steps adopted.

TAR evaluation

Mantel test comparisons between the distance matrices derived from the ARISA and 454 sequencing data sets (Sørensen index), highlighted significant correlations between these two data sets ($0.28 < r < 0.62$, $P < 0.001$). These significant correlations showed that the two data sets were congruent and could be compared with one another (data not shown).

DNA fingerprinting (ARISA) and meta-barcoding were compared for their assessment of community turnover rate (z) using the Sørensen dissimilarity index on binary data. The estimated z ranged from 0.007 to 0.046, from 0.009 to 0.063, from 0.009 to 0.08 and from 0.013 to 0.09 in the Brittany, Burgundy, Landes and South-East regions, respectively (Figure 2). Except in Landes, the estimated z with the ARISA data set was always lower than the values obtained with the NGS data set, irrespective of the similarity thresholds used. In addition, z increased significantly with the sequence similarity threshold used for the clustering of OTUs, except between 95 and 97% similarity. Furthermore, the coefficients of variation of z were systematically smaller with the NGS data set than with the ARISA data set in every region and for every clustering level except in Landes at 80% similarity. Indeed, in Landes, the coefficients of variation for z ranged from 26 to 46% with NGS and were 40% with ARISA. For the three other regions, the coefficients of variation of z ranged from 8 to 12% with NGS and from 10 to 17% with ARISA. The same trend was observed when the four regions were compared with one another,

except at the 85% similarity threshold where z was higher in the South-East than in Brittany, Landes and Burgundy (Figure 2). No significant differences were observed between these three regions. A similar trend was observed with the ARISA data set. Finally, no significant differences between regions were observed at a similarity threshold of 85%.

Variance partitioning of community assembly

The relative importance of the sets of spatial variables, land-use and pedo-climatic characteristics on variations in bacterial community assembly was used to compare the DNA meta-barcoding and fingerprinting approaches for their capacity to help understanding observed biological patterns.

The amount of variance in bacterial community assembly explained by spatial and environmental parameters ranged from 19.5% to 23.0%, 23.3% to 31.6%, 7.2% to 29.8% and 23.7% to 30.6% in Brittany, Burgundy, Landes and South-East, respectively, according to the molecular approach adopted (DNA fingerprinting vs DNA meta-barcoding) (Figure 3). In each region, slightly higher amounts of community variance were explained for the ARISA data set than for the NGS data set. In addition, similar amounts of community variance were explained between the different thresholds of sequence similarity used for OTUs clustering in the NGS approach with the Sørensen index (from 80 to 97% similarity levels, data not shown) in a given region.

All groups of explanatory variables (pedo-climatic characteristics, land-use or spatial variables) were selected with the ARISA and NGS data sets, independently of the Sørensen index (Figure 3). However, the land-use and spatial variables were not selected in Landes with NGS, whereas they were selected with ARISA. Comparison of variance partitioning of the ARISA and NGS data sets with the Sørensen index

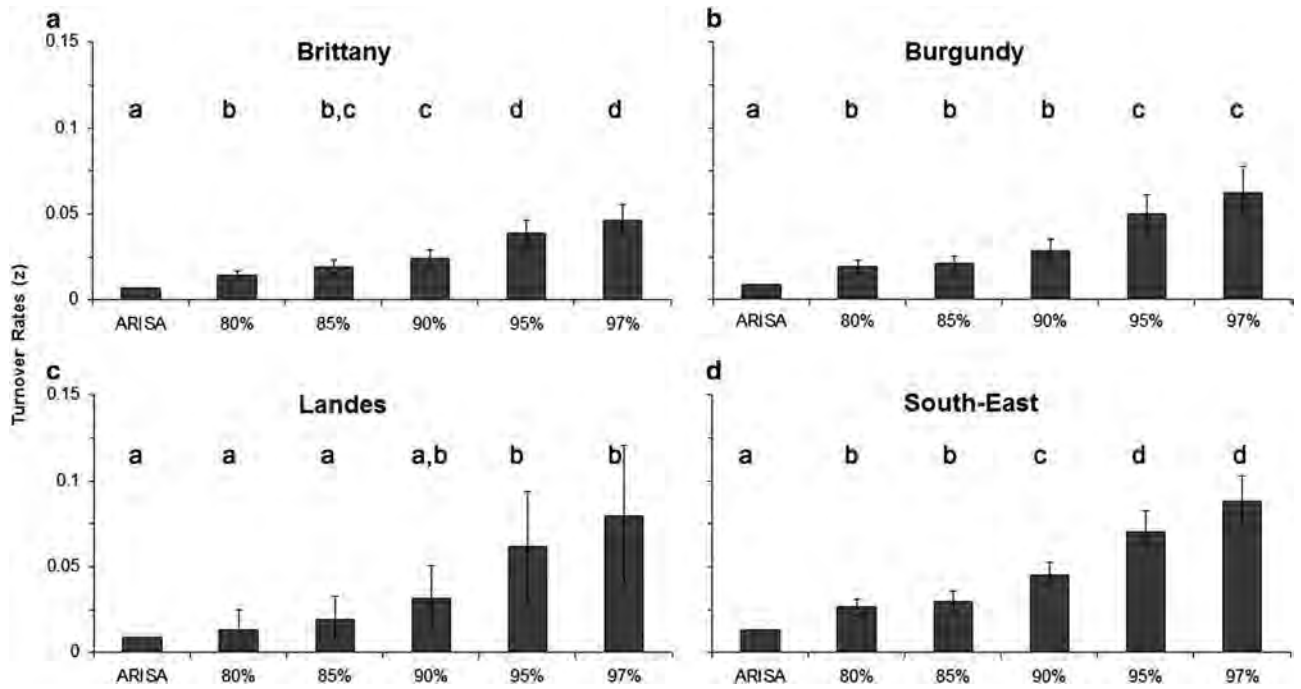


Figure 2 Comparison of ARISA and NGS approaches for the estimation of soil bacterial community turnover rate. Comparisons of each approach were performed within each region: (a) Brittany; (b) Burgundy; (c) Landes; (d) South-East. Percentages indicate the level of similarity considered in the NGS approach. Letters indicate significant differences between turnover rates in each region at the 5% probability level.

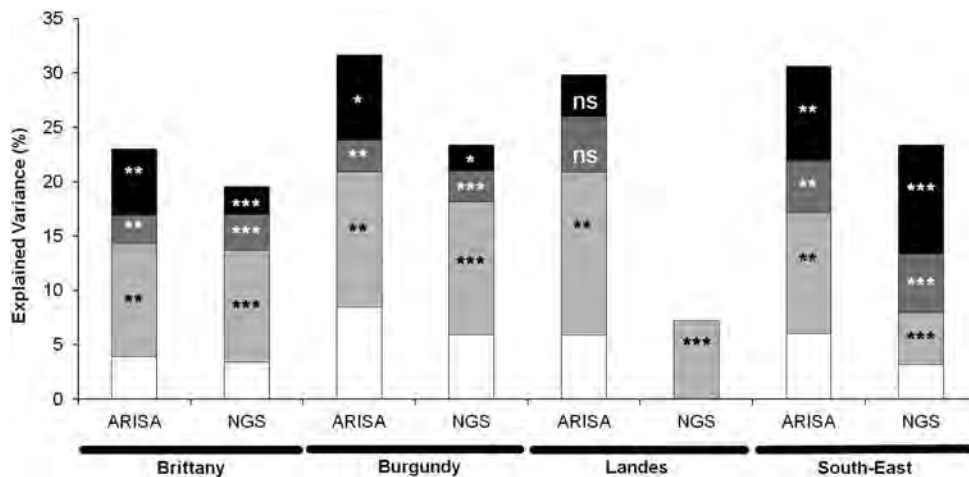


Figure 3 Variance partitioning of community composition with ARISA and NGS approaches in Brittany, Burgundy, Landes and South-East. Four groups of variables were considered: pedo-climatic characteristics (light grey), land-use (dark grey), spatial descriptors (black), which stand for residual spatial autocorrelation and interactions between the three sets of filters (white). Interactions between the three groups of variables were estimated but could not be tested for their significance. Percentages indicate the level of similarity considered in the NGS approach. Significance codes: ns: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

revealed slightly different results for some regions when groups of explanatory variables were ranked according to their marginal effect. In the Brittany, Burgundy and Landes regions, pedo-climatic characteristics explained the highest amount of variance (10%, 12.5% and 7–15%, respectively) whatever the molecular approach or dissimilarity index whereas spatial variables accounted for a systematically higher variance with the ARISA data set than with the NGS approach (Figure 3). For the South-East region, the use of ARISA or NGS data sets led to different hierarchies in the groups of explanatory variables. With the ARISA data set, pedo-climatic characteristics explained the highest amount of variance (11%), followed by spatial variables (8.7%) and land-use (4.8%). With the NGS data set, spatial

variables were the most important (9.2%), followed by land-use and pedo-climatic characteristics (5.5% and 4.8%, respectively).

DISCUSSION

The four regions considered in this study followed a gradient of environmental heterogeneity: low level (Landes region), medium level (Brittany and Burgundy) and high level (South-East) as observed by means of the dispersion of sites according to regions in the multivariate analysis on mixed data. Most of these differences were related to variability of environmental parameters reported in the literature to be involved in shaping soil microbial diversity: land-use (Drenovsky *et al.*, 2010), soil characteristics (texture, pH, P content and C:N ratio;

Fierer and Jackson, 2006; Dequiedt *et al.*, 2011; Lienhard *et al.*, 2013) and to a lesser extent climate (Fierer and Jackson, 2006). This environmental heterogeneity had been shown to affect the soil bacterial community turnover rate (z , slope of the TAR in the similarity distance–decay relationship, Harte *et al.*, 2009; Ranjard *et al.*, 2013). These four regions therefore provided a valid sampling design for determining whether DNA meta-barcoding, as compared with molecular fingerprinting, would provide a more accurate estimation of TAR and a better understanding of the processes involved in bacterial community assembly on a broad scale.

A preliminary step in the comparison of DNA meta-barcoding and fingerprinting approaches was to post-process the data according to molecular analysis steps specific to each method. For DNA fingerprinting, this was handled by setting a fixed number of OTU_{bin} to be considered during the band profiles analysis (Ranjard *et al.*, 2001, 2013). For the NGS approach, methodological biases (for example, PCRs or sequencing errors), which might generate OTUs of low abundance and equally represented across samples, were removed by bioinformatic filters (Quince *et al.*, 2011) and by two post-processing steps. These steps allowed the preservation of information on the ‘rare biosphere’ while removing artifacts (Kunin *et al.*, 2010; Terrat *et al.*, 2012; Supplementary Table S5), conversely to the classical post-processing step (removal of all low abundant OTUs). As the data were analyzed as presence–absence data, these steps seemed a relevant consensus to avoid up-weighting the importance of rare and specific OTUs in the data set (Van Dorst *et al.*, 2014; Zinger *et al.*, 2014), while conserving a fine description of bacterial community assembly (Supplementary Table S3). These post-processing steps led to the conservation of at least 6000 sequences per sample, which was higher than those used in a recent study (*ca* 4000 reads per sample, Zinger *et al.*, 2014). This study demonstrated that community z was weakly affected by the sequencing depth per sample unless it was shallow (<500 sequences), and that it was independent of the number of reads between samples (Zinger *et al.*, 2014).

In this context, DNA fingerprint and meta-barcoding approaches were compared for their estimation of soil bacterial community z . Both approaches associated with the Sørensen index demonstrated significant z estimates, which were in accordance with those classically observed in the literature with fingerprinting or low-depth sequencing data (Green *et al.*, 2004, 2006; Horner-Devine *et al.*, 2004; Bell *et al.*, 2005; Woodcock *et al.*, 2006; Martiny *et al.*, 2011; Ranjard *et al.*, 2013; Zinger *et al.*, 2014). Interestingly, the z estimates observed with the DNA meta-barcoding approach were higher than those obtained with ARISA, although the OTU_{bin} richness in the ARISA data set was similar to the OTU richness in the NGS data set for low clustering thresholds (from 80 to 90% similarity). Moreover, the coefficients of variation of z estimated with the DNA meta-barcoding approach were systematically smaller than with the ARISA, thereby highlighting that the z estimates obtained by DNA meta-barcoding approach are more accurate. These differences in z estimates and coefficients of variation can easily be explained: ARISA involves the analysis of the length polymorphism of the intergenic spacer between the 16S and 23S ribosomal genes (IGS) that can be considered less informative than DNA meta-barcoding, which assesses the sequence (size and nucleotide composition) (Ranjard *et al.*, 2000; Terrat *et al.*, 2012). This accuracy of the NGS approach in describing community assembly may reduce the similarity between sites, leading to higher estimates of community turnover. From an ecological viewpoint, DNA fingerprint and meta-barcoding approaches displayed a similar capacity to discriminate samples and both demonstrated the significant spatial structuration of soil bacterial communities in the different regions

considered. In addition, both methods revealed similar trends between the regions for the hierarchy of soil bacterial community z : Brittany < (Burgundy, Landes) < South-East, in agreement with the study by Van Dorst *et al.* (2014), which demonstrated the similar capacity of ARISA and DNA meta-barcoding to discriminate sites at a local spatial scale. This underlines the value of both methods in demonstrating ecological trends for soil bacterial communities. Nevertheless, the DNA meta-barcoding approach provides a finer description of soil bacterial community composition than ARISA, which supports the hypothesis that the turnover rates estimated derived from the latter approach would be underestimated. One advantage of the DNA meta-barcoding approach is that a description of community assembly can be drawn from the construction of similarity clusters (OTUs) at various thresholds of sequence similarity. Here, soil bacterial community z increased significantly with increasing clustering thresholds (80 to 97%). This increase was mainly related to an increase of OTU richness from low to high clustering thresholds, in agreement with Harte *et al.* (2009). Interestingly, the values of z obtained for high clustering thresholds were comparable to those observed for macroorganisms (MacArthur and Wilson, 2001; Horner-Devine *et al.*, 2004), high thresholds that are considered to reflect taxonomic levels classically used in macroecological studies (Rosselló-Mora and Amann, 2001). These findings raise insights for microbial ecologists studying the spatial structuring of soil microbial communities as they contradict the classically observed positive relationship between community turnover rate (z) and organism body size (Hillebrand *et al.*, 2001; Drakare *et al.*, 2006), and also suggest that soil microbial communities may display strong spatial structuration.

Meta-barcoding and fingerprint approaches were compared for their ability to correlate pedo-climatic, land-use and spatial variables with soil bacterial community distributions in a distance-based redundancy analysis (Sørensen index). These environmental sets of variables captured significant amounts of community variance for both data sets (8 to 35%), which were within the range of those reported in the literature for bacteria (Martiny *et al.*, 2011; Hanson *et al.*, 2012). Surprisingly, slightly higher amounts of community variance were explained for ARISA data. Van Dorst *et al.* (2014), in contrast, had reported the opposite trend. This might be related to differences in the spatial scales investigated (broad vs local scale) and in data processing (OTU_{bin} definition and bioinformatics steps for OTUs clustering).

The variance partitioning approach also allowed the comparison of the relative importance of pedo-climatic, land-use and spatial variables in shaping bacterial community assembly according to the molecular approach. The same hierarchy was observed for the different sets of variables in Burgundy and Brittany, but not in the Landes and South-East regions. Indeed, in the South-East region, spatial variables became the main driver of community assembly according to the DNA meta-barcoding approach. This result suggests that dispersal limitations may be as important as selection in shaping bacterial community assembly (Martiny *et al.*, 2011) because this process could lead to a spatial autocorrelation of bacterial communities between sites. This hypothesis was supported by Bryant *et al.* (2008) who highlighted the primary importance of elevation in limiting the dispersal of *Acidobacteria*. In the South-East region, elevation was the most important spatial variable explaining bacterial community assembly. Altogether, this suggests that use of the DNA meta-barcoding approach could lead to reexamination of the relative importance of the processes shaping soil microbial diversity on a broad scale.

CONCLUSION

Although DNA fingerprinting and meta-barcoding are both relevant to demonstrate the spatial structuration of soil microbial communities through significant TAR, the DNA meta-barcoding approach provides a finer description of soil bacterial community assembly. It also provides a more accurate estimation of community turnover rates. Considering the processes shaping soil bacterial diversity, both identical conclusions were not systematically obtained, suggesting that DNA meta-barcoding approach may lead to reexamine their relative importance. Nevertheless, this should be tested for other soil microbial communities like fungi. In addition, in a context of up-scaling studies in microbial biogeography, the meta-barcoding approach may help to identify not only the scales at which soil microbial communities are structured, but also the processes or the filters shaping their diversity at each spatial scale.

Data archiving

The raw data sets are available on the EBI database system under project accession number PRJEB6290.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Similar Processes but Different Environmental Filters for Soil Bacterial and Fungal Community Composition Turnover on a Broad Spatial Scale

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Abstract

Spatial scaling of microorganisms has been demonstrated over the last decade. However, the processes and environmental filters shaping soil microbial community structure on a broad spatial scale still need to be refined and ranked. Here, we compared bacterial and fungal community composition turnovers through a biogeographical approach on the same soil sampling design at a broad spatial scale (area range: 13300 to 31000 km²): i) to examine their spatial structuring; ii) to investigate the relative importance of environmental selection and spatial autocorrelation in determining their community composition turnover; and iii) to identify and rank the relevant environmental filters and scales involved in their spatial variations. Molecular fingerprinting of soil bacterial and fungal communities was performed on 413 soils from four French regions of contrasting environmental heterogeneity (Landes < Burgundy < Brittany < South-East) using the systematic grid of French Soil Quality Monitoring Network to evaluate the communities' composition turnovers. The relative importance of processes and filters was assessed by distance-based redundancy analysis. This study demonstrates significant community composition turnover rates for soil bacteria and fungi, which were dependent on the region. Bacterial and fungal community composition turnovers were mainly driven by environmental selection explaining from 10% to 20% of community composition variations, but spatial variables also explained 3% to 9% of total variance. These variables highlighted significant spatial autocorrelation of both communities unexplained by the environmental variables measured and could partly be explained by dispersal limitations. Although the identified filters and their hierarchy were dependent on the region and organism, selection was systematically based on a common group of environmental variables: pH, trophic resources, texture and land use. Spatial autocorrelation was also important at coarse (80 to 120 km radius) and/or medium (40 to 65 km radius) spatial scales, suggesting dispersal limitations at these scales.

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Introduction

For over two centuries, biogeographical studies have been carried out on macroorganisms and have provided a better understanding of species distribution, extinction and interactions [1–2]. For microorganisms, the first biogeographic postulate was developed by Baas Becking in 1934 [3]: "Everything is everywhere, *but*, the environment selects" suggesting that microbial "species" may be everywhere due to huge dispersal potentials, but that their abundances are constrained by contemporary environmental context, which may be especially true at broad

spatial scales (spatial scales larger than 100 km² are considered as broad in this study). The number of studies in microbial biogeography has increased exponentially over the past decade thanks to new molecular tools applicable in routine on wide scale sampling networks constituted of several hundreds of samples [4–5]. These studies revealed that soil microorganisms are not strictly cosmopolitan since their distributions are systematically heterogeneous and structured into biogeographical patterns [2], [6–9].

One way to discriminate the spatial processing of microbial diversity is to evaluate either the Taxa-Area Relationship (TAR),

i.e. the accumulation of new taxa with increasing sampling area, or the Distance-Decay Relationship (DDR), *i.e.* the rate of change in compositional similarity with increasing distance. [10–11]. Although significant TAR and DDR have recently been demonstrated for both soil fungal [12] and bacterial [8], [11], [13–14] communities, the relative importance of the ecological processes shaping these communities is still under debate. Therefore, it needs to be more deeply considered at the community level. According to Vellend [15], four processes are involved in shaping microbial community composition: selection, dispersal, ecological drift and speciation. Speciation is difficult to consider at the community level because the molecular markers used to discriminate microbial taxa mainly target highly conserved regions (*e.g.* ribosomal genes) with low mutation rates. The stochastic demographic processes underlying ecological drift are also difficult to consider since it remains a challenge to fully characterize demographic evolutions within complex microbial communities in environmental samples. Consequently, most biogeographical studies have focused on environmental selection and dispersal limitations, the latter leading to a spatial autocorrelation between sites independently of environmental factors. Numerous studies have identified environmental selection as relevant in shaping soil bacterial community composition [8–9], [16–24]. Conversely, dispersal limitation is still under debate regarding the high dispersal potentials of microorganisms and because some environmental variables always remain unmeasured. Nevertheless, recent publications also suggest that bacteria may be dispersal limited [9], [21–23] or that part of soil bacterial communities is endemic [17]. As regards soil fungi, the relevance of environmental selection and dispersal limitations has been demonstrated at the community level and for ectomycorrhizal groups [16], [19], [22], [24–29]. Nevertheless, most of these studies were performed on different sampling designs with different molecular techniques. Only few studies have investigated such processes for both soil fungal and bacterial communities simultaneously to compare their biodiversity turnover. Most of them were performed in particular ecosystems and lead to diverging conclusions: Pasternak et al [18] concluded that bacterial and fungal communities were primarily shaped by environmental selection rather than dispersal limitations at the scale of the Israeli desert. On the contrary, Talbot et al. [27] highlighted a strong endemism for fungi in pine forests and Hovatter [22] suggested that the ecological processes shaping soil bacterial community could differ at a local scale due to the presence/absence of a particular plant. Altogether, this suggests that environmental heterogeneity may determine the relative importance of the ecological processes at work and therefore affect the distance-decay relationship for both soil bacteria and fungi [9–11], also suggested by other macrobial studies [30–31]. The comparison of different microbial communities along different levels of environmental heterogeneity may therefore help to reach a consensus.

Both selection and dispersal are based on the various ecological attributes of soil bacteria and fungi in terms of soil colonization, dispersal forms, trophic requirements, biological interactions and adaptation to environmental conditions, together with stochastic factors. Consequently, studies focusing particularly on soil bacteria or soil fungi have identified numerous environmental filters involved in shaping these particular communities but no consensus could be reached regarding microbial community as a whole on broad spatial scales. The filter most frequently identified for bacteria is soil pH [5], [17], [20], [23–24], [32–34] and it is commonly assumed that this is an important driver for fungal communities [18], [24]. Soil texture and carbon content have also been identified as important filters for bacteria [6], [22–23], [33–

34]. Similarly, the quality of soil organic matter, represented by the C:N ratio, and the amount of N were shown to have a significant effect on the abundance and composition of bacterial and fungal communities [24], [34–35]. These edaphic factors are often considered as the main determinants of bacterial diversity since the importance of climate may vary across biomes at a continental scale [5], [12], [16]. Land-use, agricultural practices, and plant community composition are also important filters for both bacteria and fungi on a wide scale [18], [19], [22–24], [36–38]. Therefore, this suggests that common filters determine the composition of both bacterial and fungal communities, but they still need to be ranked according to their relative importance to reach a consensus. This may be achieved by comparing bacterial and fungal communities over regions contrasted in terms of habitat heterogeneity but also with wide ranges of variations for the identified filters.

The objectives of this study were: i) to examine the spatial structuring of bacterial and fungal communities on a broad spatial scale; ii) to investigate the relative importance of environmental selection and spatial autocorrelation in determining the community composition turnover of these communities; and iii) to identify and rank the relevant environmental filters and scales involved in their spatial variations. To attain these objectives, four regions in the RMQS data set (“Réseau de Mesures de la Qualité des Sols” = French Monitoring Network for Soil Quality, recovering 2,200 soils over the whole of France) were selected along a gradient of environmental heterogeneity, representing a total of 413 soils. This gradient was chosen in order to confront the community composition turnover rates of bacterial and fungal communities to soil habitat heterogeneity [9]. Bacterial and fungal communities were characterized by Automated RISA fingerprinting of soil DNA. Community composition turnover (z) was estimated by means of a similarity DDR using an exponential model as suggested by Harte et al [39–40] for microorganisms. Together with this measure of community composition turnover over broad spatial scales, the initial similarity of communities was evaluated [30]. It represents the variability of community composition at finer spatial scales. High initial similarity corresponds to low local variability. The relative influence of environmental selection and spatial autocorrelation was investigated through a variance partitioning approach involving pedoclimatic characteristics and land-use and spatial variables (geographic coordinates and Principal Coordinates of Neighbour Matrices; PCNM), respectively.

Methods

Soil samples

Soil samples were provided by the Soil Genetic Resource Conservatory (platform GenoSol, http://www.dijon.inra.fr/plateforme_genosol, [41]) and obtained from the soil storage facility of the RMQS (“Réseau de Mesures de la Qualité des Sols” = French Monitoring Network for Soil Quality). The RMQS database consists of observations of soil properties on a 16-km regular grid across the 550000 km² French metropolitan territory and was designed to monitor soil properties [42]. The baseline survey consisting of 2,200 sites (each corresponding to a composite soil sample constituted of 25 soil cores) was completed in 2009. The sites were selected at the centre of each 16×16-km cell. In this study, we focused on a subset of 413 sites from the RMQS data set. The samples were organized into four regions: Brittany (131 sites), Burgundy (109 sites), Landes (52 sites) and South-East (121 sites, Fig. 1A) which are contrasted in terms of soil type, land-use (coarse level of the CORINE Land Cover classification; IFEN, [PLOS ONE | www.plosone.org](http://</p>
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www.ifen.fr; 7 classes: forest, crop systems, grasslands, particular natural ecosystems, vineyards/orchards, parkland and wild land), climate and geomorphology (Table S1). Within a region, sites were separated by 16 km at least. For each soil, the pedo-climatic characteristics considered were particle-size distribution, pH in water (pH_{water}), organic carbon content (C_{org}), N content, C:N ratio, soluble P contents, CaCO_3 and exchangeable cations (Ca, K, Mg), sum of annual temperature ($^{\circ}\text{C}$) and annual rainfall (mm). Physical and chemical analyses were performed by the Soil Analysis Laboratory of INRA (Arras, France) which is accredited for such analyses by the French Ministry of Agriculture.

Bacterial and fungal community fingerprinting

Soil DNA extraction. For each soil sample, the equivalent of 1.5 g of dry soil was used for DNA extraction, following the procedure optimized by platform GenoSol [35]. Briefly, extraction buffer (100 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl and 2% (w/v) SDS) was added to the sample in the proportion 3:1 (v/w), with two grams of glass beads (106 μm diameter) and eight glass beads (2 mm diameter) in a bead-beater tube. All beads were acid washed and sterilized. The samples were homogenized for 30 s at 1600 rpm in a mini bead-beater cell disruptor (Mikro-dismembrator, S. B. Braun Biotech International), incubated for 30 min at 70°C in a water bath and centrifuged for 5 min at 7000 g and room temperature. The supernatant was collected, incubated on ice with 1/10 volume of 3 M potassium acetate (pH 5.5) and centrifuged for 5 min at 14000 g. DNA was

precipitated with one volume of ice-cold isopropanol and centrifuged for 30 min at 13000 rpm. The DNA pellet was washed with ice-cold 70% ethanol and dissolved in 100 μL of ultra pure water. For purification, aliquots (100 μL) of crude DNA extracts were loaded onto PVPP (polyvinyl polypyrrolidone) minicolumns (BIORAD, Marne la Coquette, France) and centrifuged for 4 min at 1000 g and 10°C . This step was repeated if the eluate was opaque. The eluate was then collected and purified for residual impurities using the GeneClean Turbo kit as recommended by the manufacturer (Q Biogene, France).

PCR conditions. The bacterial ribosomal IGS was amplified using the PCR protocol described in Ranjard et al [43]. 12.5 ng of DNA was used as the template for PCR volumes of 25 μL . The fungal ribosomal ITS was amplified using the primer set ITS1F/ITS4-IRD800 (5'-CTTGGTCATTTAGAGGAAGTAA-3'/5'-IRD800-TCCTCCGCTTATTGATATGC-3'). 20 ng of DNA was used as the template for PCR volumes of 25 μL with the following PCR conditions: denaturation at 95°C for 3 min, 35 cycles of 30 s at 95°C , 45 s at 55°C and 1 min at 72°C , and a final elongation of 7 min at 72°C . The primer T_m was the same for bacterial IGS and fungal ITS. Every PCR products were purified using the MinElute Kit (QIAGEN, Courtaboeuf, France) and quantified using a calf thymus DNA standard curve.

ARISA fingerprinting conditions. 2 μL of the PCR product was added to deionized formamide and denatured at 90°C for 2 min. Bacterial and Fungal ARISA fragments were resolved on 3.7% polyacrylamide gels under denaturing conditions as

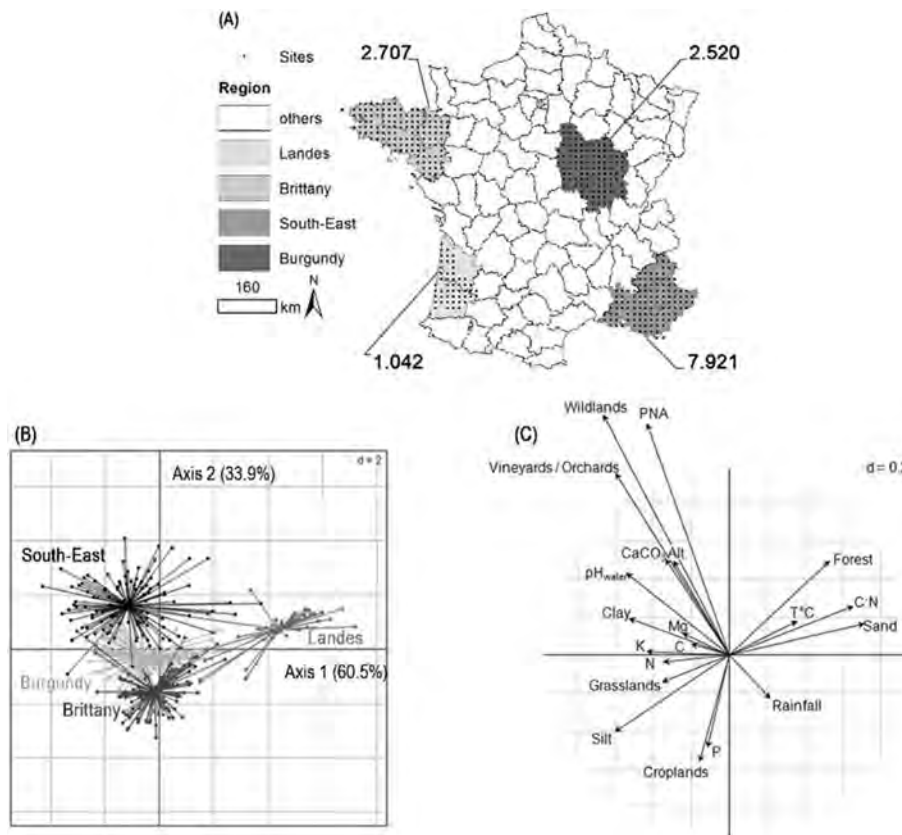


Figure 1. Comparison of the regions considered on the basis of their soil habitats. A. Soil average dissimilarity of soil habitat for the different regions (number linked to the corresponding region) and position of sites; B. Between group analysis of soil habitats according to the region; C. Correlation circle of the variables defining soil habitat in the between group analysis. The length of the arrow corresponds to the Pearson's correlation coefficient for quantitative variables and to the correlation ratio for qualitative variables. Symbols: Alt.: Elevation; $T^{\circ}\text{C}$: Sum of annual temperatures; P_{ass} : Assimilable P; C:N: Carbon to Nitrogen ratio; C_{org} : Organic Carbon content. doi:10.1371/journal.pone.0111667.g001

described in Ranjard et al [9] on a LiCor DNA sequencer (ScienceTec).

Image analysis. The data were analyzed using the 1D-Scan software (ScienceTec), converting fluorescence data into electrophoregrams, where peaks represented PCR fragments (100 to 110 peaks retained per sample, the resolution limit to avoid considering background noise). The height of the peaks was calculated in conjunction with the median filter option and the Gaussian integration in 1D-Scan, and represented the relative proportion of the fragments in the total products. Lengths (in base pairs) were calculated by using a size standard with bands ranging from 200 to 1659 bp. The data were then converted into a contingency table with prepRISA package in R.

Statistical analyses

Characterization of habitat variability and average dissimilarity across regions. Habitats were compared between regions in a Hill & Smith multivariate analysis [44] using the ade4 package in R [45–46]. The analysis was applied to pedoclimatic characteristics, land-use and geomorphology, by centering and scaling the quantitative variables, and converting the qualitative ones into weighted binary variables (weight equal to $1/n$; n is the number of classes for the qualitative variables). Differences between these regions were examined by between group analysis and tested by applying a Monte-Carlo permutation test (1000 permutations). The average dissimilarity between soil habitats was determined by transposing a method based on the dissimilarity matrix for communities [47] to soil habitat. The dissimilarity matrix for soil habitat was derived from the site coordinates in the Hill & Smith analysis, following equation 1 [9]:

$$D_{i,j} = \frac{ED_{i,j}}{ED_{\max}} \quad (1)$$

Where $D_{i,j}$ and $ED_{i,j}$ are the dissimilarity and the Euclidean distance between sites i and j , respectively. ED_{\max} is the maximum Euclidean distance observed between sites. The average dissimilarity between soil habitats was then calculated as follows [47]:

$$\bar{D}_{\text{habitat}} = \frac{1}{n} \sum_{h=1}^{n-1} \sum_{i=h+1}^n D_{h,i}^2 \quad (2)$$

\bar{D}_{habitat} is the average dissimilarity between soil habitats of soil habitat and n the number of sites in the region.

Evaluation of the similarity distance-decay relationship and initial similarity of Bacterial and Fungal community composition. The *similarity distance-decay* relationship was estimated as proposed by Harte et al [39] for organisms with large populations per taxa. From this relationship, the community composition turnover rates (z) for bacterial and fungal communities composition were derived as described in Ranjard *et al.* [9] following the method and the exponential model (equation 3) proposed by Harte et al [39–40] for microorganisms.

$$\log_{10}(\chi_d) = (-2 * z) * \log_{10}(d) + b \quad (3)$$

Where χ_d is the observed Sørensen's similarity between two soil samples that are d meters apart from each other; b is the intercept of the linear relationship and z the turnover rate of the community composition. The z estimate and its 95% confidence interval were derived from the slope ($-2*z$) of the relationship between similarity and distance by weighted linear regression. The overlap of the 95% confidence intervals was used to test for significant differences

in community composition turnover rates between regions or between bacteria and fungi. The initial similarity of community composition was taken as the average similarity between sites 16 km apart and the 95% confidence intervals of the mean were determined [30].

Variance partitioning of community composition variations according to environmental filters and space. The relative importance of spatial variables, pedoclimatic characteristics and land-use in determining community composition turnover was tested by db-RDA [47–48]. Quantitative data were centered and scaled. Spatial variables were constructed from site coordinates (x , y , elevation) to reveal potential spatial trends at scales larger than the region, and of Principle Coordinates of Neighbour Matrices [49] in each region. The PCNM approach creates independent spatial descriptors that can be introduced in canonical analysis models to consider the spatial autocorrelation between sites in the model [49]. PCNMs with a significant Moran index ($P < 0.001$) were selected. Land-use corresponded to the Corinne Land Cover classes recoded into dummy variables. Pedo-climatic characteristics consisted of climate and all the physico-chemical variables except sand. The most parsimonious model was obtained by forward selection from null to full model in two steps: a first step for selecting environmental variables and a second step for selecting the relevant PCNMs. Then, the pure effects of each set of filters or each individual filter were tested with an anova-like permutation test for canonical analyses (anova.cca function in vegan package, [50]). The PCNMs approach does not provide directly the range of the spatial descriptor. Nevertheless, Bellier et al [51] demonstrated that kriging approach could be applied to PCNMs to estimate their spatial range. Consequently, when PCNMs were selected in the most parsimonious model, their ranges were determined by standard kriging techniques (ordinary kriging with a Gaussian model). The hierarchy of these filters must nevertheless be considered with caution due to the small amounts of variance explained by each one. Land-use was not included in the filter-ranking since it corresponded to a set of categories and a global “land-use” category was already taken into account in the processes section. Maps of soil fungal community structure variations are provided as (Fig. S1, mapping methodology is described in the legend).

Results

Heterogeneity of soil habitat

The four regions were selected for their contrasting environmental heterogeneity as demonstrated by the between group analysis (Fig. 1B) and comparison of the calculated average dissimilarity between soil habitats (\bar{D}_{habitat}) (Fig. 1A and C). Multivariate analysis revealed a clear discrimination of the four regions on the first and second axes (Monte-Carlo permutation test, $P < 0.001$): Landes was significantly discriminated from Brittany, Burgundy and South-east on the first axis and these three regions were discriminated from each other on the second axis. In addition, the environmental variability strongly differed between the four regions as demonstrated by the dispersal of sites in the factorial map. Sites from Landes were less dispersed on the factorial map than the sites from Brittany or Burgundy, which were less dispersed than sites from South-East. The calculated \bar{D}_{habitat} ranged from 1.042 in the Landes to 7.921 in the South-East, with intermediate values for Burgundy and Brittany (2.520 and 2.707, respectively; Fig. 1A). It provided the same discrimination between regions. Fig. 1C shows that the four regions could mainly be distinguished according to land-use (*e.g.*: 86% of the

Landes sites are forest sites), a restricted set of soil physico-chemical characteristics (sand and silt contents, pH_{water} and CaCO_3 content, assimilable P content and organic matter quality as measured by C:N ratio) and by differences in elevation. Climatic conditions did not play a significant role in regional discrimination.

Distance-Decay Relationship for bacterial and fungal soil communities

Bacterial and fungal community similarity decreased with increasing distance in each region (Fig. 2). Community similarity was systematically higher for bacteria than for fungi in all regions at small or large distances. For bacteria, the linear regression model was highly significant in each region ($P < 0.001$, Fig. 2) except in Landes where it was just below the significance threshold of 5% ($P < 0.02$). For fungi, the linear regression model was highly significant in each region ($P < 0.001$) except Landes (Fig. 2).

Community composition turnover rates were derived from the parameters of the linear regression. The community composition turnover rates for the bacterial and fungal communities ranged from 0.006 to 0.013 (Table 1). No significant differences were highlighted between these organisms when the community composition turnover rates were compared within each region.

When the community composition turnover of bacterial or fungal communities was compared between regions, a significant difference was only found between Brittany and the South-East ($P < 0.05$) for bacteria.

The initial similarity was always higher for bacterial communities than for fungal communities within each region, ranging respectively from 54.4% to 61.4% and from 39.7% to 43.1%. The initial similarity of the bacterial community in Landes was significantly lower than in the other regions, which did not differ from each other. For fungi, the initial similarities in Landes and Brittany were similar and significantly lower than those in Burgundy and the South-East.

These results were confirmed by a covariance analysis comparing the models between organisms within a region and between regions for a given organism (data not shown).

Variance partitioning of community composition variations

The relative importance of the sets of spatial variables, land-use and pedo-climatic characteristics on variations in bacterial and fungal community composition was tested by db-RDA using the Sørensen index (Fig. 3). The amount of variance in bacterial and fungal community composition explained by the three sets of filters

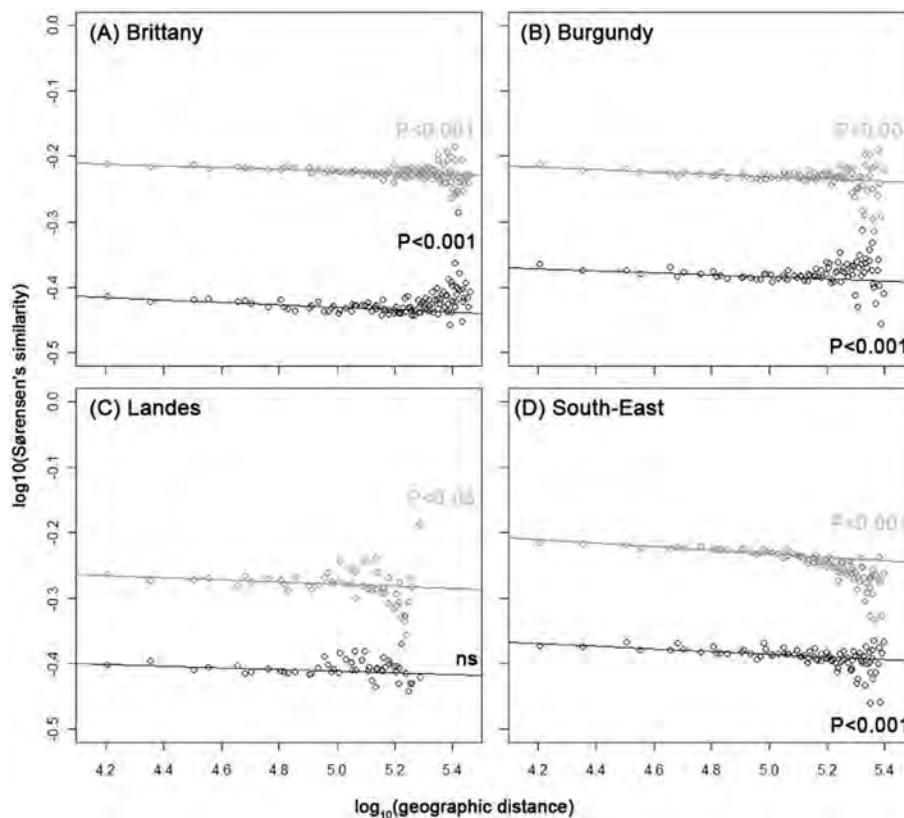


Figure 2. Distance-Decay Relationships for bacteria and fungi. Each panel correspond to a region: Brittany (A), Burgundy (B), Landes (C) and South-East (D) Points the average Sørensen's similarity between sites for each distance class. Lines represent the regression model based on the whole set of paired comparisons; for bacteria (grey) and fungi (black). The equations for the regression models were as follows: **(A) Brittany:** Bacteria***: $\log_{10}(\text{Sørensen's similarity}) = -0.014 \times \log_{10}(\text{geographic distance}) - 0.156$; Fungi***: $\log_{10}(\text{Sørensen's similarity}) = -0.017 \times \log_{10}(\text{geographic distance}) - 0.350$; **(B) Burgundy:** Bacteria***: $\log_{10}(\text{Sørensen's similarity}) = -0.018 \times \log_{10}(\text{geographic distance}) - 0.144$; Fungi***: $\log_{10}(\text{Sørensen's similarity}) = -0.015 \times \log_{10}(\text{geographic distance}) - 0.316$; **(C) Landes:** Bacteria*: $\log_{10}(\text{Sørensen's similarity}) = -0.017 \times \log_{10}(\text{geographic distance}) - 0.198$; Fungi^{ns}: $\log_{10}(\text{Sørensen's similarity}) = -0.012 \times \log_{10}(\text{geographic distance}) - 0.357$; **(D) South-East:** Bacteria***: $\log_{10}(\text{Sørensen's similarity}) = -0.027 \times \log_{10}(\text{geographic distance}) - 0.101$; Fungi***: $\log_{10}(\text{Sørensen's similarity}) = -0.019 \times \log_{10}(\text{geographic distance}) - 0.298$. A graph with points representing all paired-comparisons between sites as points can be found in Figure S2. Significance of the model is indicated as an exponent for each organism: ns: not significant; $P < 0.05$: *; $P < 0.01$: **; $P < 0.001$: ***. doi:10.1371/journal.pone.0111667.g002

Table 1. Regression parameters of the Distance-Decay Relationships for Bacteria and Fungi.

Region	Parameter	Organism	Estimate	95% Confidence interval
Brittany (131)	Z	Bacteria	0.007	[0.005; 0.009]
		Fungi	0.009	[0.006; 0.011]
	Initial similarity	Bacteria	61.4%	[60.4%; 62.3%]
		Fungi	38.5%	[37.7%; 39.4%]
Burgundy (109)	z	Bacteria	0.009	[0.006; 0.012]
		Fungi	0.008	[0.003; 0.012]
	Initial similarity	Bacteria	61.4%	[60.4%; 62.5%]
		Fungi	43.1%	[42.0%; 44.3%]
Landes (52)	z	Bacteria	0.009	[0.001; 0.016]
		Fungi	0.006	[-0.001; 0.013]
	Initial similarity	Bacteria	54.4%	[52.9%; 56.0%]
		Fungi	39.7%	[38.4%; 40.9%]
South-East (121)	z	Bacteria	0.013	[0.011; 0.016]
		Fungi	0.009	[0.006; 0.013]
	Initial similarity	Bacteria	60.9%	[59.9%; 61.9%]
		Fungi	42.4%	[41.5%; 43.3%]

The number of observations per region is provided in brackets beside the name of the region. The community composition turnover rate (z) and the initial similarity are derived from the slope of the regression (-2z) and the mean of similarity at 16 km; respectively. The statistical comparison between region and organism was performed by examining the overlap of the 95% confidence intervals of turnover rates or initial similarities.
doi:10.1371/journal.pone.0111667.t001

ranged from 17% to 32%. The significance of the interactions between the three sets of filters could not be tested but always explained a small amount of the total variance (from 1.2% to 8.4%).

Among the sets of filters, soil pedo-climatic characteristics were the main contributor to variations in bacterial and fungal community composition. Spatial variables systematically explained a lower amount of variance than pedo-climatic characteristics, but a higher amount than land-use for both bacteria and fungi except for bacteria in Landes region where neither spatial variables nor land-use were significant.

The amount of variance explained by pedo-climatic characteristics for bacteria or fungi was always significant, ranging from 5% to 15%, and was similar between regions. Nevertheless, within each region, the amount of variance explained by soil pedo-

climatic characteristics was always higher for bacteria than for fungi (Fig. 3).

Spatial variables significantly explained part of the community composition variations in all regions except for bacteria in Landes region. When significant, spatial variables represented from 3% to 9% of the total variance and were of the same order of magnitude both between bacteria and fungi and between regions (Fig. 3).

Similarly, land-use explained a significant amount of community composition variations in all regions except for bacteria in Landes region. The amount of explained variance ranged from 2.6% to 6.5% of the total variance. Within each region, land-use explained similar amounts of bacterial and fungal community variations when significant. Between regions, amounts of variance explained by land-use were similar for both bacteria and fungi (Fig. 3).

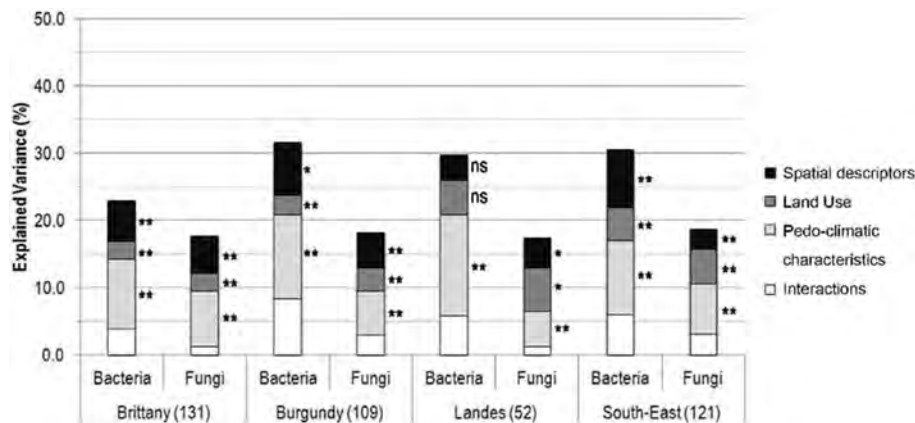


Figure 3. Variance partitioning of bacterial and fungal community composition. The number indicated in brackets corresponds to the number of samples for the region. Significance levels: ns: not significant; P<0.05: *; P<0.01: **; P<0.001: ***.
doi:10.1371/journal.pone.0111667.g003

Hierarchy of environmental filters

Within the sets of soil pedo-climatic characteristics and spatial variables, the pure effects of individual filters on bacterial and fungal community structure are presented in Figure 4. These pure effects account for relatively small proportions of the total variance (from 0.7% to 6.5%) because of the large number of filters explaining the total variance of bacterial or fungal community compositions. Regarding the pedo-climatic characteristics, the significant filters for soil bacterial communities in Brittany were first pH and secondly N content. For fungi, these were the quality of organic matter resource as indicated by the selection of N content, C_{org} content and C:N ratio. The least significant filters corresponded to soil texture and other soil nutrients for bacteria (clay and silt contents, Mg and $CaCO_3$ concentrations) and fungi (clay content, K and P concentrations, Figure 4). In Burgundy, as in Brittany, the soil bacterial and fungal communities were principally affected by pH. Beside pH, bacterial community composition was shaped by the quality of organic matter resource (N content, C_{org} content, C:N ratio) followed by clay content and annual rainfall. The fungal community composition was shaped by C:N ratio only. The only filter that had a significant effect in the Landes region, on both soil bacteria and soil fungi, was the C:N ratio. The pH was the most important filter, followed by clay content and K concentration, for bacteria in the South-East. In this region, N content and K concentration were more important filters for fungi than pH and clay content.

The spatial variables corresponded to the sites coordinates and 16 significant PCNM eigenfunctions, each representing a different spatial scale of analysis (coarse, medium and fine scales, Figure 4). Longitude, latitude or altitude coordinates did not influence community composition except latitude for fungi in Burgundy region (Figure 4). PCNMs representing spatial structures of 80–120 km radius explained significant amounts of variance in the composition of bacterial and fungal communities in Brittany and South-East. This highlighted that these communities were spatially structured at a coarse spatial scale. Similarly, PCNMs representing spatial structures of 40–65 km radius explained significant amounts of variation in the composition of bacterial community

in Brittany and of fungal community in Burgundy, South-East and Landes. This showed the spatial structuration of these communities at medium spatial scales. PCNMs accounting for fine scale variables were neither significant for bacteria nor fungi in any region.

Discussion

The four regions were selected to challenge the hypothesis that different levels of environmental heterogeneity, *i.e.* different habitat diversity and fragmentation for soil bacteria and fungi, results in different community composition turnovers [9], [14] and to compare their determinism. The multivariate analysis and the calculated $\bar{D}_{habitat}$ both highlighted a significant gradient in environmental heterogeneity following the sequence: Landes < Burgundy ≤ Brittany << South-East. The four regions were mainly discriminated by environmental variables already demonstrated to influence soil microbial community abundance and diversity, such as land-use [52] and soil characteristics (texture, pH_{water} , P content and C:N ratio; [6], [14], [33], [35]). Among these four regions, Landes and South-East represent two extremes of environmental heterogeneity. Landes was distinct in having sites with conifer forest on acidic soils and low altitudinal variations. This specificity may turn Landes into an outlier for its environmental variability. On the other hand, South-East is characterized by a strong altitudinal gradient and a mosaic of land-use. In comparison, Burgundy and Brittany were also characterized by a mosaic of land-use but were more marked by croplands than South-East and less by forests. In addition, these two regions presented intermediate soil types regarding Landes and South-East: silty-clay calcic soils and silty acidic soils, respectively; associated to intermediate levels of organic Carbon content. Altogether, these observations highlight that these four regions allow the consideration of a large range of environmental conditions associated to different levels of environmental heterogeneity. Therefore, community similarity turnover rates can be confronted to environmental heterogeneity and their comparison may lead to a consensus regarding the environmental filters shaping soil microbial communities.

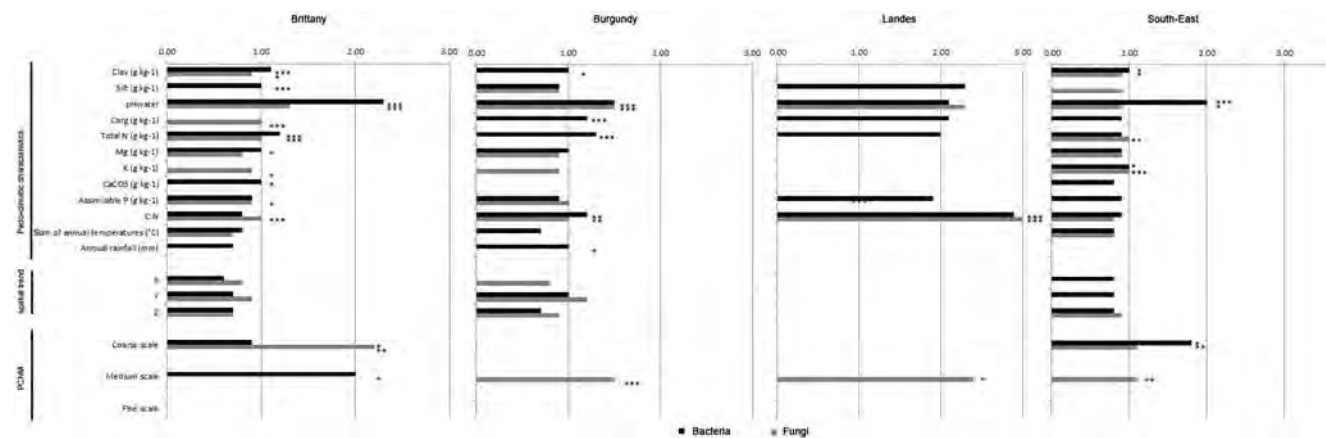


Figure 4. Variations of microbial communities partitioned according to edaphic variables and space. For each organism and region, only variables retained in the most parsimonious model are presented and their pure effect is tested by a permutation test. Significance levels are: $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. Missing values or variables indicate that the variable was not retained in the model. Sand was removed prior to model evaluation since it was represented by the opposite of the sum of silt and clay content. Rainfall: Sum of annual rainfall (mm). Temperature: Sum of annual temperature ($^{\circ}C$). Spatial components were summarized according to the scale considered: trend (x, y and z coordinates), coarse, medium or fine. The interval in brackets indicates the numbers of PCNMs retained in the model for each scale. The proportion of variance for each scale was determined as the sum of the pure effects of each PCNM when these were significant. Coarse, medium and fine scales correspond to PCNM with a spatial range of 80 to 120 km, 40 to 65 km and less than 40 km; respectively.
doi:10.1371/journal.pone.0111667.g004

In all regions, except Landes, the soil bacterial and fungal communities were spatially structured as indicated by the significant DDR. This supports that the concept of DDR for soil microorganisms may be generalized, as suggested by several other studies [1], [9], [12], [24], [53]. The estimated bacterial and fungal community composition turnover rates ranged from 0.006 to 0.013. This is in agreement with the recent community composition turnover rates observed for soil microorganisms [10], [14], [24]. However, the turnover rates estimates were low. This could be due to technical limitations; particularly the low taxonomic resolution of DNA fingerprinting. Species variations were aggregated by the DNA fingerprints into few dominant bands, which precluded the accumulation of new minor species with increasing distance [54–55]. This would be supported by the higher community composition turnovers observed in Zinger [24] with the high resolution level provided by the pyrosequencing approach. The sampling design could also have led to low estimates of the community composition turnover rate. Indeed, lower composition turnover rates are commonly observed in regions composed of contiguous habitats [13] with gradual variations of habitat characteristics across sites and higher rates of community composition turnover have been observed at finer taxonomic levels and spatial scales [56].

Beside these technical points, community composition turnover rates were very close within each region for bacteria and fungi. This observation was in agreement with maps of bacterial and fungal communities' structures which revealed large patches of *ca.* 100–140 km radius (see Fig. 2 in [7] for bacteria and Fig. S1 for fungi). Altogether, this indicated a low-level of aggregation of both communities on a broad spatial scale [57]. This observation would suggest that differences in terms of biological and ecological features (habitat characteristics, colonization modes, trophic requirements, biotic interaction, dormancy; [6], [58–59]) would not lead to different rates of community composition turnover on a broad spatial scale. This would be supported by strong biotic interactions between bacterial and fungal communities highlighted by a significant correlation between bacterial and fungal communities' compositions. Nevertheless, this correlation was much lower than that between each community composition and environmental variables (data not shown). This strong dependency to environmental conditions resulted in a trend towards higher turnover rates in regions with higher $\bar{D}_{habitat}$. This would corroborate our first hypothesis that the microbial DDR is positively related to environmental heterogeneity. This observation was more marked and significant for bacterial communities but only a tendency for fungal communities. Although this conclusion is based on analyses of only 4 regions, it is in agreement with Ranjard *et al.* [9] who demonstrated a correlation between habitat heterogeneity and turnover rate for soil bacterial communities on a broad spatial scale, and with other microbial and macrobial studies [24], [30–31], [60].

The differences between the sensitivities of soil bacteria and fungi to environmental heterogeneity could be due to the ecology of soil fungi [58] at the spatial scale of this study. Nevertheless, according to Peay *et al.* [25–26], ectomycorrhizal fungi are spatially structured at very fine spatial scales. This would suggest that these organisms are strongly dependent on environmental conditions but that the grain size of this study (252 km²) did not allow perceiving this dependence. This hypothesis would be supported by the estimated similarity of the microbial communities at a local spatial scale (initial similarity, [30]). Indeed, the initial similarity was systematically higher for bacterial than for fungal communities. According to Morlon *et al.* [57], this would indicate that fungal communities are more aggregated than bacterial communities, *i.e.*

more variable than bacterial communities, at a local spatial scale. This would lead to the conclusion that fungi are somehow more dependent on environmental conditions than bacteria at a local scale, in agreement with Peay *et al.* [25–26] and the numerous biological interactions they are involved in.

The observed patterns and significant turnover rates result from different ecological processes [8] which may shape differently bacterial and fungal community composition on a broad spatial scale according to their relative importance. Recent studies have demonstrated that the main processes involved in increasing or decreasing community composition turnover rate are environmental selection and dispersal, respectively [8–9]. Dispersal is commonly supposed, but not empirically demonstrated to be high for microbes [61]. The maintenance of significant turnover rates, as recorded in our study, thus requires environmental selection to be high and dispersal not infinite. This hypothesis was tested by partitioning the β -diversity variations of microbial communities within regions. This partition was made according to filters involved in environmental selection: soil pedo-climatic characteristics and land-use; and to spatial variables characterizing spatial autocorrelation unexplained by the environmental variables. The residual spatial autocorrelation could result from dispersal limitations, but also from unmeasured spatially autocorrelated environmental variables despite the extensive number of environmental variables considered in this study. The amount of explained variance ranged from 23% to 32% for bacteria and from 17.5% to 18.8% for fungi. These values are within the range reported in the literature for the whole communities of bacteria [8], [14] and fungi [25–26], [62].

Soil characteristics accounted for higher amounts of variance, for both bacteria and fungi, than spatial variables. This supports the idea that soil microbial communities are primarily affected by environmental selection and secondly by other processes leading to spatial structuration independently of the environment [2], [7–8], [38]. More precisely, environmental selection was mainly driven by pedo-climatic characteristics, which accounted for larger amounts of variance than land-use. This is in agreement with several studies conducted on a broad spatial scale evidencing the higher dependency of bacterial and fungal communities on soil physico-chemical characteristics [18], [38], [63] than on land-use. Nevertheless, the amount of variance explained by pedo-climatic characteristics was higher for bacteria than for fungi, highlighting the greater effect of soil habitat on shaping the bacterial community. This observation is supported by the higher diversity and reactivity of the soil bacterial community to changes in surrounding conditions which, in turn, leads to a community structure that is better fitted to the habitat characteristics [13], [58]. On the contrary, land-use explained a higher amount of fungal community variance than of bacterial community variance. This latter could be due to plant-soil microbe interactions resulting from the type of vegetative cover [19], [38], [52], [64] as well as from human activities, especially agricultural or industrial practices [35]; [37] potentially affecting fungi more strongly than bacteria. This difference would be in agreement with a highly patchy distribution of soil fungi at a local scale as suggested above by the low level of initial similarity. Altogether, these results support the postulate of Baas-Becking “the environment selects” in summarizing microbial biogeography.

Nevertheless, variance partitioning also highlighted that bacterial and fungal communities are spatially structured independently of environmental characteristics since spatial variables accounted for a significant amount of community variance. A hypothesis in explaining this spatial autocorrelation is that soil bacterial and fungal communities might be dispersal limited, even if this result

may also be related to unmeasured, spatially structured, environmental variables. This hypothesis would be supported by the study of Ranjard et al [9] demonstrating significant turnover rates of bacterial diversity in fully homogenous regions and by the consideration of several soil physico-chemical characteristics in the variance partitioning approach of the present study. Nevertheless, this must be confirmed by more in-depth studies since few evidence remains to date on the shaping of bacterial and fungal communities by limited dispersal [8], [17], [26–27], [65]. Comparisons have indicated that fungi tend to be less dependent on spatial variables than bacteria. This result is surprising since fungi are demonstrated to be dispersal limited [27] and bacteria are generally expected to disperse over larger distances, even wider than our regional scale [14], [66]. It was estimated in the literature that 10^{18} viable bacteria were transported annually in the atmosphere between continents [61]. Moreover, particular fungal populations have been demonstrated to disperse over short distances, *e.g.* ectomycorrhiza at the “plant island scale” [25–26], or to be endemic [27]. On the other hand, variance explained by spatial variables was similar in each region for fungi, whereas it followed the environmental heterogeneity gradient: Landes < Brittany < Burgundy < South-East for bacteria. This observation supports the hypothesis that bacteria are more sensitive to geomorphology and that the presence of natural barriers (*e.g.* mountains), or a higher fragmentation of landscape in the South-East, due to a mosaic of agricultural and natural plots [9] may lead to significant spatial variations.

Our last objective was to identify and rank the environmental filters and the spatial variables involved in environmental selection process and spatial autocorrelation of bacteria and fungi. To do so, the environmental and spatial filters selected in the variance partitioning approach were ranked according to their pure effect on bacterial and fungal community composition turnover. First, environmental selection was mainly based on soil characteristics but not on climatic conditions. This is in accordance with previous studies highlighting the weak influence of climate on soil microbial diversity on a broad spatial scale [6–7], [35], [37], [67], [68]. Nevertheless, recent studies also demonstrated strong differences between bacterial communities across different biomes, especially cold deserts *versus* temperate biomes and hot deserts, suggesting that climate may play a role [32] at very large spatial scales. Overall, the main soil characteristics identified as important filters for soil bacterial and fungal community composition were pH, trophic resources (N, C, K, P contents; C:N ratio) and texture (clay, silt content). These findings are consistent with the literature where i) pH is regularly identified as the main filter for both soil bacteria [6], [16–17] and fungi [25], [62], ii) soil organic C and N contents and the C:N ratio constitute important components of microbial niches [18], [32], [35], [58], and iii) texture determines the size and stability of soil micro-habitats [18], [69], [70]. The hierarchy of these filters depended on the type of organisms and the regions. The above-described hierarchical sequence (pH > trophic resources ≥ texture) was observed for three of the four regions, but not for Landes. In the Landes region, the filter variability is low and environmental selection is basically driven by organic matter quality (C:N ratio). This is related to the large number of conifer forests sites leading to soil organic matter with a high C:N ratio and strong recalcitrance to microbial decomposition. A strong selection of particular populations with enzymatic ability to transform this organic matter is occurring in such soils [58], which is deeply influencing the corresponding community structure. Comparison of the overall filter hierarchies for bacteria and fungi in a given region did not reveal any discrepancy for the primary drivers. The only differences were observed for secondary

filters, including C:N ratio and mineral nutrients (such as K and P) for fungi, and clay and N content for bacteria. This is consistent with the well-known dependency of the soil fungal community on soil P content [34] and soil organic matter quality [58]. As regards bacteria, clay content is positively correlated with the biotic capacity of soil as well as its indigenous bacterial diversity by enhancing the level of protection of the soil habitat and the retention of nutrients [69–70]. Second, regarding spatial autocorrelation, different scales were derived from the hierarchy of spatial variables and their range. The main scale identified was the coarse scale (80 to 120 km radius), for both bacteria and fungi in Brittany and South-east regions. This spatial scale is smaller than the one at which soil habitat changed on the RMQS Network (150 to 470 km, [9]), whereas it is in agreement with the large patches obtained by mapping the bacterial and fungal community structure over these regions [7] and Fig. S1). In addition, finer spatial scales (medium scales; 40 to 65 km radius) were also identified as significant in Burgundy, Landes and South-East for fungi and in Brittany for bacteria. Altogether, this scale dependency would support a hypothesis for the dispersal limitation of bacterial and fungal communities [33]. This scale dependency is in agreement with Martiny et al [14] for bacteria and is supported by the observations of Peay et al [25–26] on soil fungi. As in other studies at continental scales [14], this highlights the importance of considering multiple scales to better understand microbial ecology.

Altogether, our study demonstrated the spatial structuring of soil bacterial and fungal communities on local to coarse scales, which was based on environmental selection and on an unexplained spatial autocorrelation that could be related to limited dispersal. Selection and spatial autocorrelation were shown to have a similar influence on soil bacteria and soil fungi but the filters involved could differ depending on the environmental heterogeneity. Nevertheless, the comparison of bacterial and fungal communities helped to propose a primary consensus regarding the environmental filters shaping soil microbial community composition as a whole: Land-Use and pH are the primary filters, followed by trophic resources quantity (organic carbon content and nitrogen content) and then quality (C:N ratio). The results of this study increase our knowledge on the effects of soil habitat and provide insights in the scale at which dispersal may occur according to the ecological attributes of bacteria and fungi. However further investigations, based on up-scaling approaches, are now required to: i) provide a direct measurement of bacterial cells dispersal which is now crucial to demonstrate limited dispersal of bacteria; ii) identify the filters operating at each spatial scale. Especially, these upscaling approaches could be associated to high-throughput sequencing to achieve a finer resolution on the communities. This would improve our ability to sustainably manage soil biodiversity.

Supporting Information

Figure S1 Maps of interpolated MULTISPATI scores for the first three MULTISPATI axes (columns) and for the four geographical regions (rows). Each map was generated as described in Dequiedt et al (2009) and corresponds to the spatial synthesis of the F-ARISA genetic structure of indigenous fungal communities from the corresponding soils sampled in the four regions of France. Colours on the map are proportional to the score of each soil sample on each MULTISPATI axis following the scale provided at the bottom of the figure. Below each column, the empirical variogram is provided for each MULTISPATI axis. (DOC)

Figure S2 Distance-Decay Relationship for bacteria and fungi. Each panel correspond to: (A–D): **Bacteria** in Brittany,

Burgundy, Landes and South-East; **(E–H): Fungi** in Brittany, Burgundy, Landes and South-East. Points represent paired-comparisons between sites and line the linear model. The equations for the regression models were as follows: **(A)** $\log_{10}(\text{Sørensen's similarity}) = -0.014 \times \log_{10}(\text{geographic distance}) - 0.156$; **(B)** $\log_{10}(\text{Sørensen's similarity}) = -0.018 \times \log_{10}(\text{geographic distance}) - 0.144$; **(C)** $\log_{10}(\text{Sørensen's similarity}) = -0.017 \times \log_{10}(\text{geographic distance}) - 0.198$; **(D)** $\log_{10}(\text{Sørensen's similarity}) = -0.027 \times \log_{10}(\text{geographic distance}) - 0.101$; **(E)** $\log_{10}(\text{Sørensen's similarity}) = -0.017 \times \log_{10}(\text{geographic distance}) - 0.350$; **(F)** $\log_{10}(\text{Sørensen's similarity}) = -0.015 \times \log_{10}(\text{geographic distance}) - 0.316$; **(G)** $\log_{10}(\text{Sørensen's similarity}) = -0.012 \times \log_{10}(\text{geographic distance}) - 0.357$; **(H)** $\log_{10}(\text{Sørensen's similarity}) = -0.019 \times \log_{10}(\text{geographic distance}) - 0.298$. Signifi-

cance of the model is indicated as an exponent for each organism: ns: not significant; $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. (DOCX)

Table S1 Summary statistics of regions characteristics. PNE: Particular Natural Ecosystems, SE: standard error of the mean. (DOC)

Author Contributions

Conceived and designed the experiments: NCPB SD NPAS CJ DA LR PL. Performed the experiments: NCPB ML. Analyzed the data: NCPB SD JT PP. Contributed reagents/materials/analysis tools: NCPB SD JT ML. Wrote the paper: NCPB LR.

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Pyrosequencing evidences the impact of cropping on soil bacterial and fungal diversity in Laos tropical grassland

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Abstract It is widely assumed that agricultural practices have a major impact on soil living organisms. However, the impact of agricultural practices on soil microbes is poorly known, notably for species richness, evenness, and taxonomic composition. The taxonomic diversity and composition of soil indigenous microbial community can be assessed now using pyrosequencing, a high throughput sequencing technology applied directly to soil DNA. Here, we studied the effect of agriculture management on soil bacterial and fungal diversity in a tropical grassland ecosystem of northeastern Laos using 454 pyrosequencing of 16S and 18S rRNA genes. We studied soil microbial diversity of agricultural soils 3 years after conversion from native grasslands. We compared five systems: one tillage, two no-tillage rotational, one no-tillage improved pasture, and one natural grassland. Our results show first that compared to the natural grassland, tillage decreases fungal richness and diversity by -40% and -19% ,

respectively and increases bacterial richness and diversity by $+46\%$ and $+13\%$, respectively. This finding evidences an early impact of agricultural management on soil microbial diversity. Such an impact fits with the ecological concept of "intermediate perturbation"—the hump-backed model—leading to classify agricultural practices according to the level of environmental stress they generate. We found also that land use modified soil microbial taxonomic composition. Compared to the natural pasture, tillage decreased notably the relative abundance of *Actinobacteria* (by -6%), *Acidobacteria* (by -3%) and *Delta-proteobacteria* (by -4%) phyla, and by contrast increased the relative abundance of *Firmicutes* (by $+6\%$), *Gamma-proteobacteria* (by $+11\%$), and *Chytridiomycota* ($+2\%$) phyla. We conclude that soil microbial diversity can be modified and improved by selecting suitable agricultural practices. Moreover no-till systems represented intermediate situations between tillage and the natural pasture and appear therefore as a fair trade-off between the need for agriculture intensification and soil ecological integrity preservation.

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Acid savannah

1 Introduction

Among human activities, agricultural practices strongly affect soil microbial communities by changing the physical and chemical characteristics of the soil in which microorganisms live, thereby affecting their abundance, diversity, and activity (Kladivko 2001; Govaerts et al. 2007). Agricultural inputs (e.g., organic amendments, mineral fertilizers, and pesticides), crop rotation, and plant diversity affect soil microorganisms in different ways (Bunemann et al. 2006; Nicolardot et al. 2007; Pascual et al. 2013). However, in conventional agriculture, tillage generally has the greatest

impact on biological properties since physical disturbance changes soil water content, temperature, aeration, and the degree of mixing of crop residues within the soil matrix (Kladivko 2001; Six et al. 2006). Tillage also reduces soil macroaggregate content (Tivet et al. 2013), which provides an important microhabitat for microbial populations (Ranjard and Richaume 2001). Based on the principle of minimal soil disturbance, no-till farming systems have been widely adopted in large-scale mechanized agriculture to prevent soil erosion and decrease production costs (Derpsch et al. 2010). Combined with soil cover (mulch) and diversified crop rotation, no-till systems are also being advocated over tillage for enhancing soil health and long-term crop productivity (Govaerts et al. 2007).

To date, the effects of agricultural practices and, more widely, of cropping intensity were mostly evaluated on soil microbial abundance and structure by using classical tools based on the cultivability of microorganisms as well as on their physiological and biochemical properties (Kladivko 2001; Kandeler 2007). However, these techniques were strongly limited in their sensibility and exhaustivity to give an accurate overview of quantitative and qualitative modifications of soil microbial communities (Maron et al. 2010). The recent development of culture-independent molecular tools, and especially of high throughput sequencing technology (pyrosequencing), allows obtaining thousands of sequences from a single soil DNA sample which may help better assessing the huge diversity of soil microbial communities (Roesch et al. 2007; Terrat et al. 2011). Metagenomic analysis should facilitate the deciphering of taxonomic and functional assemblages of indigenous communities in natural environments, together with their roles in the biological functioning of ecosystems (Maron et al. 2010). To date, this approach has been poorly used to evaluate the impact of agricultural practices on soil microbial communities, hence limiting our interpretation of soil microbial taxonomic diversity changes, and their significance in terms of soil ecological status and potential functioning in agrosystems.

In a previous study, we showed that tillage systems and cover crops rapidly affected soil microbial abundance and genetic structure in a tropical grassland ecosystem of north-eastern Laos (Lienhard et al. 2013). However, the genotyping techniques used were unable to accurately characterize the full diversity of the telluric microbial communities. Consequently, they could not narrow the knowledge gap concerning the distribution and diversity (in terms of species richness and evenness) of indigenous microbial species in response to soil disturbance and cropping intensity.

In this study, our objective was to deepen the effect of agricultural management on soil microbial diversity by using a metagenomic approach. More precisely, our study aims at making an inventory of the diversity of both soil bacterial and fungal communities using new generation sequencing

technology on soil DNA. For this, we compared soils coming from five contrasted land use management systems (one tillage-based and two no-till rotational cropping systems, one no-till improved pasture and the natural surrounding pasture), 3 years after the conversion of native grassland into agricultural land. Soil bacterial and fungal diversity were evaluated by 454 pyrosequencing of 16S and 18S rRNA genes, respectively. The analysis of microbial diversity changes, and notably the changes in particular taxonomic groups' distribution was used to evaluate agricultural systems effect on soil ecological status.

2 Material and methods

2.1 Experimental site and land use management

Experiments were conducted in Poa village (latitude, 19°33'N; longitude, 102°59'E) at 1,130 m AMSL. The climate is tropical and mountainous with a 6-month (April–September) wet and hot season and a 6-month dry season including 3 months of cold. The mean annual precipitation is 1,400 mm. The soils at the site are red clayey oxisols (USDA classification). We studied five land use management systems representing a decreasing gradient of cropping intensity and soil disturbance (Table 1): one tillage-based rotational system (CT), based on soil ploughing and repeated human interventions (e.g., sowing, manual weeding, and fertilizer application); two no-till rotational systems (NT1 and NT3) with limited soil disturbance (no-tillage) but also including frequent agricultural operations (e.g., rolling, spraying, sowing, and fertilizer application); one intensively grazed no-till and mono-specie improved pasture (ImpP); and a natural unfertilized and barely grazed grassland (PAS). The annual crop treatments (CT, NT1, and NT3) were selected from the split-split plot experimental design described in Lienhard et al. (2013). The pasture treatments (ImpP and PAS) were taken from surrounding fields (Fig. 1).

2.2 Soil sampling and chemical analysis

The soil was sampled at 0–10-cm depth, on the 29th of June 2010, 40 days after maize sowing. A composite sample was made of a pool of five subsamples taken in the diagonal section of the plot. For annual crop systems, soil was sampled on the maize row to avoid a possible “cover crop” effect under no-till systems. Soil chemical analyses were done by the INRA laboratory in Arras, France. Soil texture was measured by sieving methods (3 classes). Soil organic carbon (SOC) and total nitrogen (N) were quantified by dry combustion. Soil pH was measured in 1:5 soil/water slurry, and exchangeable bases (Ca, Mg, K, Na) were quantified using ammonium acetate reagent.

Table 1 Main land use management characteristics

Land use	Main characteristics
PAS	Native unfertilized pasture (>30 years) dominated by <i>Themeda triandra</i> , barely grazed (animal stocking rate <0.3 head/ha ⁻¹) during the rainy season, and periodically burned during the dry season
ImpP	Improved pasture of ruzi grass direct seeded (no-till) in 2007 after the chemical control of native grasses; grazed (mean animal stocking rate of 4 heads/ha ⁻¹) and fertilized ^a during the rainy season
CT	Conventional tillage: 3-year rotation of soybean (2008), rice (2009), and corn (2010) based on annual ploughing with discs, the burying of former crop residues and weeds, and mineral fertilization ^a No-till systems: similar to CT regarding crop rotation and fertilization level ^a but conducted under no-tillage, crop residue maintenance at soil surface, and the association of cover crops prior to and with main crops:
NT 1	Fm+Pp (2007), soybean+oat+buck (2008), rice+stylo (2009), corn+Pp (2010)
NT 3	Ruzi+Pp (2007), soybean+(oat+buck)+ruzi (2008), rice (+ stylo)+ruzi (2009), corn+ruzi (2010)

Main crops: rice cv. Sebota1, corn hybrid LVN10, soybean cv. Asca. Cover crops: Fm, finger millet (*Eleusine coracana* Gaern); Pp, pigeon pea (*cajanus cajan* cv. Thai); stylo, *stylosanthes guianensis* cv. CIAT 184; oat, *Avena sativa* L.; buck, buckwheat (*Fagopyrum esculentum* Moench); ruzi, ruzi grass (*Brachiaria ruziziensis* cv. ruzi)

^a Annual fertilization of 60–80–60 kg ha⁻¹ of N–P₂O₅–K₂O (32 kg N ha⁻¹ for soybean). All agricultural treatments also received an initial application of 2 Mg ha⁻¹ of locally produced lime (27 % of CaO)

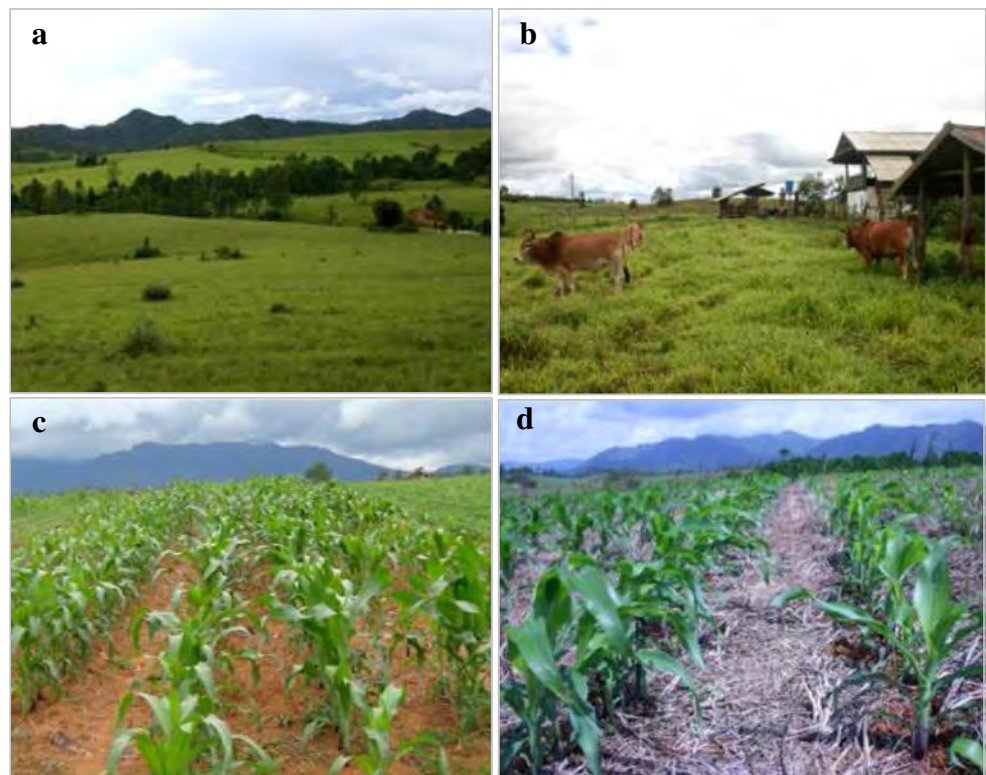
2.3 Pyrosequencing of 16S and 18S rRNA gene sequences

Microbial DNA was extracted from 2 g (dry weight) of soil using a single procedure standardized by the GenoSol platform (INRA, Dijon, France, www.dijon.inra.fr/plateforme_genosol).

Microbial diversity was estimated by 454 pyrosequencing, a molecular technique allowing a rapid and massive production of targeted DNA sequences (Maron et al. 2010). A 16S rRNA gene fragment of the appropriate size (about 440 bp) and sequence variability for 454 pyrosequencing was amplified

using the primers 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 803R (5'-CTACCNGGGTATCTAAT-3') according to a procedure described by Terrat et al. (2011). Briefly, for each soil, 5 ng of DNA was used for a 25- μ L PCR conducted under the following conditions: 94 °C for 2 min, 35cycles of 30 s at 94 °C, 52 °C for 30 s and 72 °C for 1 min, followed by 7 min at 72 °C. PCR products were purified using a MinElute gel extraction kit (Qiagen, Courtaboeuf, France) and quantified using the PicoGreen staining kit (Molecular Probes, Paris, France). Similarly, a 18S

Fig. 1 Land use management sampled in Poa experimental site. **a** Natural unfertilized pasture (PAS), **b** improved pasture of *Brachiaria ruziziensis* (ImpP), **c** maize crop under tillage (CT), and **d** maize crop under no-till system (NT1 and NT3)



rRNA gene fragment of about 350 bp was amplified using the primers FR1 (5'-ANCCATTCAATCGGTANT-3') and FF390 (5'-CGATAACGAACGAGACCT-3') under the following PCR conditions: 94 °C for 3 min, 35 cycles of 1 min at 94 °C, 52 °C for 1 min and 72 °C for 1 min, followed by 5 min at 72 °C. A second PCR of nine cycles conducted under similar conditions was then realized with purified PCR products and 10 bp multiplex identifiers added to the primers at 5' position to specifically identify each sample and avoid PCR biases. PCR products were finally purified and quantified as previously described. Pyrosequencing was then carried out on a GS Junior (Roche 454 Sequencing System).

2.4 Bioinformatic analysis of 16S and 18S rRNA gene sequences

Bioinformatic treatment was done using the GnSPipe of the GenoSol platform (INRA, Dijon, France) described by Terrat et al. (2011). Firstly, all reads were sorted according to the chosen identifiers' sequences. Then, in order to efficiently compare the datasets and avoid biased community comparisons, raw datasets which had a large number of reads were reduced by random selection close to the lowest datasets (8,000 and 2,500 reads for 16S and 18S rRNA gene sequences, respectively). Raw reads were filtered and deleted: (a) if the exact primer was not found at the beginning of the sequence, (b) if the sequences contained any ambiguity (Ns), (c) if its length was below 350 and 250 bases for 16S and 18S reads, respectively. A PERL program was then applied to obtain strict dereplication (i.e., clustering of strictly identical sequences). The dereplicated reads were then aligned using infernal alignments and clustered into molecular operational taxonomic units (MOTU) using a PERL program that clusters rare reads to abundant ones and do not count differences in homopolymer lengths. Another homemade filtering step was then applied to eliminate potential sources of errors (e.g., PCR chimeras, sequencing errors, MOTU overestimation). All single-singletons (reads detected only once and not clustered) were then checked based on the quality of their taxonomic assignments to avoid artifacts. High-quality reads were then used for taxonomy-based analysis using: (a) the Naïve Bayesian rRNA classifier of the RDP project for bacterial sequences, (b) the Basic Local Alignment Search Tool performed on a cleaned version of the Silva database (version r111 using the EMBL taxonomy) for fungal sequences. Diversity indexes were finally determined using the detected taxonomic groups at the genus level. We used the maximum number of MOTU, the Shannon (H'), and Evenness (J) indexes as indicators of soil microbial richness, diversity and structure, respectively. The raw data sets are available on the EBI database system (and Short Read Archive) under project accession number ERP002181.

2.5 Statistics

Principal component analysis (PCA), were performed using the ADE-4 package (Thioulouse et al. 1997) under R software and provided an ordination of data in factorial maps based on the scores of the first two principal components.

3 Results and discussion

3.1 Effect of agricultural management on soil chemical characteristics

Despite the limited time of cultivation at evaluation (3 years), we observed a significant early effect of agricultural systems on top soil chemical characteristics (Table 2). We recorded a rapid decrease in SOC and total nitrogen (N) under conventional tillage, with a mean loss of 25 % of SOC and total N under conventional tillage as compared to no-tilled systems, which may be related to macro-aggregate disruption, enhanced soil aeration, and the mixing of residues into the soil (Six et al. 2006). We also observed an increase in soil pH and exchangeable base content under all cultivated systems as compared to the natural pasture (Table 2) due to lime and thermophosphate supply (Table 1). However, soil exchangeable base content was much higher under no-till cultivated systems (NT1, NT3, and ImpP) than under conventional tillage (+75 % in average; Table 2), suggesting important nutrient losses by lixiviation under tillage systems. Altogether, our results are consistent with other studies comparing till vs no-till system effect on soil chemical properties at soil surface (Kladivko 2001; Govaerts et al. 2007).

3.2 Response of soil microbial diversity to land conversion to agriculture

Regardless of agricultural systems, the examination of the clustered DNA sequences revealed low microbial richness in Lao soils, with less than 550 MOTU, detected for both bacterial and fungal communities (Table 2), whereas more than 1,000 MOTU were described in other soil ecosystems for both bacterial (Acosta-Martinez et al. 2008; Terrat et al. 2012; Tripathi et al. 2012) and fungal (Buee et al. 2009) communities. This low richness could be related to the soil characteristics, since microbial diversity is strongly influenced by soil pH (Lauber et al. 2009), Al toxicity, and nutrients availability (Tripathi et al. 2012).

After 3 years of native grassland conversion to agriculture, we observed an early but significant effect of land use management on bacterial and fungal diversity (Table 2). Bacterial diversity was favoured by increased soil disturbance and cropping intensity, with values of the Shannon index (H') and richness (MOTU) decreasing along the gradient conventional

Table 2 Top soil (0–10 cm) chemical characteristics, microbial molecular abundance, and diversity according to land use management

Land use	Clay (%)	pH H ₂ O (1:5)	Soil organic content (g kg ⁻¹)	N tot (g kg ⁻¹)	C/N	Σ base ^a (cmol kg ⁻¹)	Microbial diversity					
							Bacterial			Fungal		
							MOTU	H'	J	MOTU	H'	J
PAS	57	4.9	38.6	2.6	14.8	2.1	361	3.9	0.67	392	4.8	0.80
ImpP	59	5.1	36.8	2.5	14.8	4.2	431	4.0	0.67	335	4.3	0.73
CT	55	5.0	28.9	1.9	15.0	2.5	528	4.4	0.70	236	3.9	0.72
NT1 ^b	60	5.3	39.3	2.7	14.4	4.6	507	4.3	0.70	467	4.9	0.80
NT3 ^b	63	5.2	45.1	3.1	14.5	4.3	477	4.4	0.72	341	4.5	0.78

PAS natural pasture, ImpP improved pasture, CT conventional tillage, N tot total nitrogen, C/N carbon to nitrogen ratio, MOTU molecular operational taxonomic units corresponding to richness determined at the genus level, H' Shannon index, J Evenness index

^a Sum of exchangeable bases (Ca²⁺, Mg²⁺, Na⁺, K⁺)

^b No-till system

tillage > no-till systems > improved pasture > natural pasture (Table 2). As compared to the natural pasture, conventional tillage increased H' value by +13 % (from 3.9 to 4.4) and bacterial richness by +46 % (from 361 to 528 MOTU, Table 2). Interestingly, this gradient was the opposite of the results observed for bacterial density (Lienhard et al. 2013).

According to the “hump-backed” model that links the biodiversity of a community to the intensity of its exposure to environmental stress (Giller et al. 1998; Bressan et al. 2008), our results suggest that the tillage events may at that stage represent a moderate perturbation for bacteria leading to a decrease in the competitive niche exclusion and selection mechanisms occurring between populations, and consequently to an increase in bacterial richness. In other respects, plant diversity (with no-till systems > improved pasture; Table 2) and mineral fertilization (with no-till systems and improved pasture > natural pasture) may also have contributed to increased bacterial diversity under no-till agricultural systems as compared to the native grassland due to their positive effect on soil pH and nutrient availability (Table 2) which have been shown to favour bacterial diversity (Lauber et al. 2009; Tripathi et al. 2012).

Contrary to bacterial diversity, fungal diversity was negatively affected by tillage, with lower values of richness (MOTU) and Shannon index (H') observed under conventional tillage (236 MOTU and H' of 3.9) than under no-tilled systems (MOTU ranging from 335 to 467, and H' from 4.3 to 4.9; Table 2), which might be related to the negative effect of tilling tools on fungal hyphal growth (Six et al. 2006). We found notably twofold higher fungal richness and 25 % higher H' value under no-till system 1 than under conventional tillage (Table 2). Plant diversity also appeared important in maintaining soil fungal diversity, with 40 % higher fungal richness and 14 % higher H' value observed under no till system 1 as compared to the improved pasture (Table 2), this finding being in agreement with Nishizawa et al. (2010)

who observed similar correlations between plant and fungal diversity.

Finally, although the land use management did not modify 2 years after grassland conversion into agricultural land the fungal to bacterial density ratio (Lienhard et al. 2013), it deeply impacted the fungal to bacterial diversity ratio, with the highest bacterial diversity and the lowest fungal diversity observed under conventional tillage (Table 2). Interestingly, this suggests that diversity measurements are more sensitive than density measurements to evidence early impacts of land use management on soil microbial properties.

We used the “hump-backed” relationships between biodiversity and the level of environmental stress (Giller et al. 1998; Bressan et al. 2008) to classify the agricultural systems according to their impact on soil microbial diversity (Fig. 2). Conventional tillage represented a moderate perturbation for bacteria and a high perturbation for fungi, whereas no-till and improved pasture systems represented a lower perturbation for both bacterial and fungal populations. No-till cropping systems represented intermediate situations between tillage and monospecies improved pasture systems in terms of environmental perturbation. Altogether, our results suggest the promotion of no-till systems as a fair trade-off between the need for agriculture intensification and soil biological integrity preservation.

3.3 Effect of agricultural management on soil bacterial and fungal taxonomic composition

Although the number of bacterial and fungal phyla were not significantly different between the different agricultural management (data not shown), we observed an early and strong effect on their relative abundance (Fig. 3a, b). This observation was confirmed by PCA based on taxonomic composition, with a clear discrimination of land use on the factorial maps (Fig. 3c, d). Interestingly, we observed similar gradients between bacterial diversity (Table 2) and

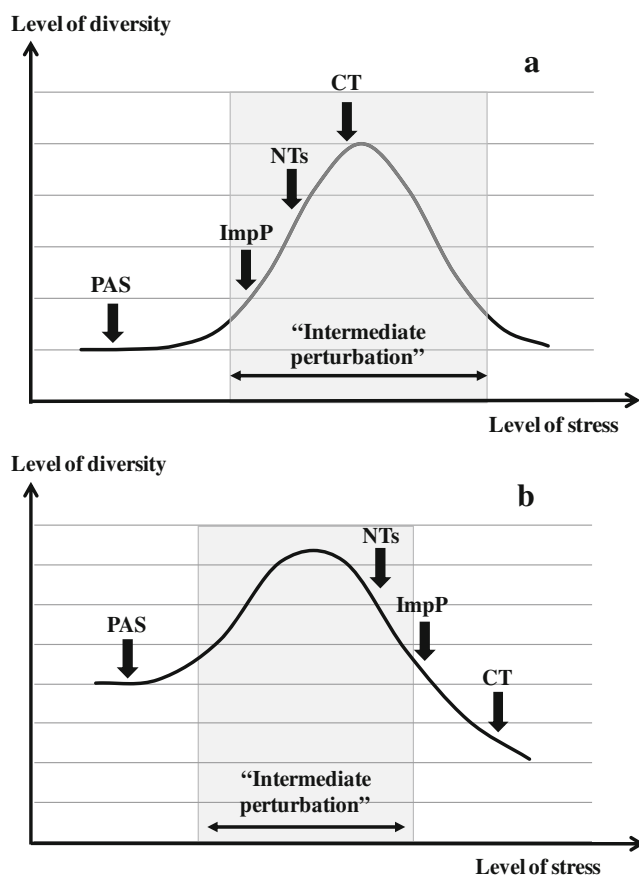


Fig. 2 Schematic representation based on the “hump-backed” model of the level of **a** bacterial and **b** fungal diversity according to the level of environmental stress induced by the different agricultural systems. Natural pasture (PAS), improved pasture (ImpP), conventional tillage (CT), no-till systems (NTs)

composition (Fig. 3c), with conventional tillage being strongly discriminated from pasture on the first PCA axis, and no-till and improved pasture representing intermediate situations. This highlights the importance of soil disturbance and cropping intensity as major drivers of soil bacterial diversity in our agricultural systems.

Regardless of land use management, the *Proteobacteria* was the dominant bacterial phyla and represented 35 to 45 % of all bacterial DNA sequences (Fig. 3a). Compared to the natural pasture, we observed a decrease of this phyla (by -6 %) under conventional tillage, which may be explained by a reduced soil organic carbon content (Table 2) since the *Proteobacteria* have been described as fast growing copiotrophs stimulated in C-rich environments (Bernard et al. 2007; Cleveland et al. 2007; Fierer et al. 2007; Jenkins et al. 2010). Agricultural management also affected the class distribution within this phyla, with a higher relative abundance of *Gamma-proteobacteria* (+11 %) and a lower relative abundance of *Alpha-* (-7 %), *Beta-* (-6 %), and *Delta-proteobacteria* (-4 %) observed under conventional tillage as compared to the natural pasture (Fig. 3c). In addition,

improved pasture was discriminated from the other no-till systems on the second PCA axis because of a higher relative abundance of *Beta-proteobacteria* (Fig. 1c), and notably of *Massilia* genus (25 % of all sequences), which has been described as a root colonizing bacteria stimulated by root exudates and readily degradable carbon compounds (Ofek et al. 2012). This is consistent with Wenzl et al. (2001) who found ruzi grass roots to exudate a high amount of organic acids (e.g., malate, citrate, and oxalate) as a mechanism of Al resistance.

The *Actinobacteria* was the second most abundant phyla with 30 to 40 % of all the bacterial sequences (Fig. 1a). *Actinobacteria* has been described as mainly K strategists (Bernard et al. 2007; Pascault et al. 2013), and well represented in non-disturbed grass systems (Acosta-Martinez et al. 2008; Yu et al. 2011). The high proportion of detected *Actinobacteria* in each system may consequently result from the pasture history of the site and the limited duration of cultivation (3 years) since conversion. However, as for the *Proteobacteria*, land use management shaped *Actinobacteria* distribution, with a decrease in relative abundance observed under conventional tillage as compared to Pasture (by -6 %; Fig. 3a, c). This may be explained by the particular morphology of those organisms, which are forming structures harbouring similarities with fungal hyphae (Stackebrandt et al. 1997) that make them more sensitive than other bacterial groups to physical soil disturbance.

Other bacterial phyla were also affected by land use management with a decrease in the relative abundance of *Acidobacteria* (by -3 %) observed under conventional tillage as compared to pasture, and by contrast, an increase in the relative abundance of the *Firmicutes* (by +6 %; Fig. 3a) that further distinguish conventional tillage and pasture on the first axis of the PCA (Fig. 3c). Bacteria belonging to the *Acidobacteria* phyla have been described as mainly oligotrophs (K-strategists), which utilize complex carbon substrates that are more likely to be present in the native SOM (Bernard et al. 2007; Fierer et al. 2007). In addition, *Acidobacteria* abundance has been shown to increase with soil age (Nemergut et al. 2007), which may explain their higher abundance in the undisturbed pasture treatment. By contrast, the *Firmicutes* have been described as mainly copiotrophs (Bernard et al. 2007; Cleveland et al. 2007; Fierer et al. 2007; Jenkins et al. 2010), which are, however, able to survive in adverse environmental conditions due to their ability to produce endospores (Mandic-Mulec and Prosser 2011). This may explain that their stimulation under conventional tillage represents the most disturbed and most carbon-depleted environment.

Regarding fungal taxonomic diversity, the dominant phyla were the *Ascomycota* (55–65 % of all fungal sequences; Fig. 3b), and the *Basidiomycota* (20–35 % of all fungal sequences). These two phyla mainly belong to the saprotrophic soil fungi (de Boer et al. 2005) and are classically dominant in

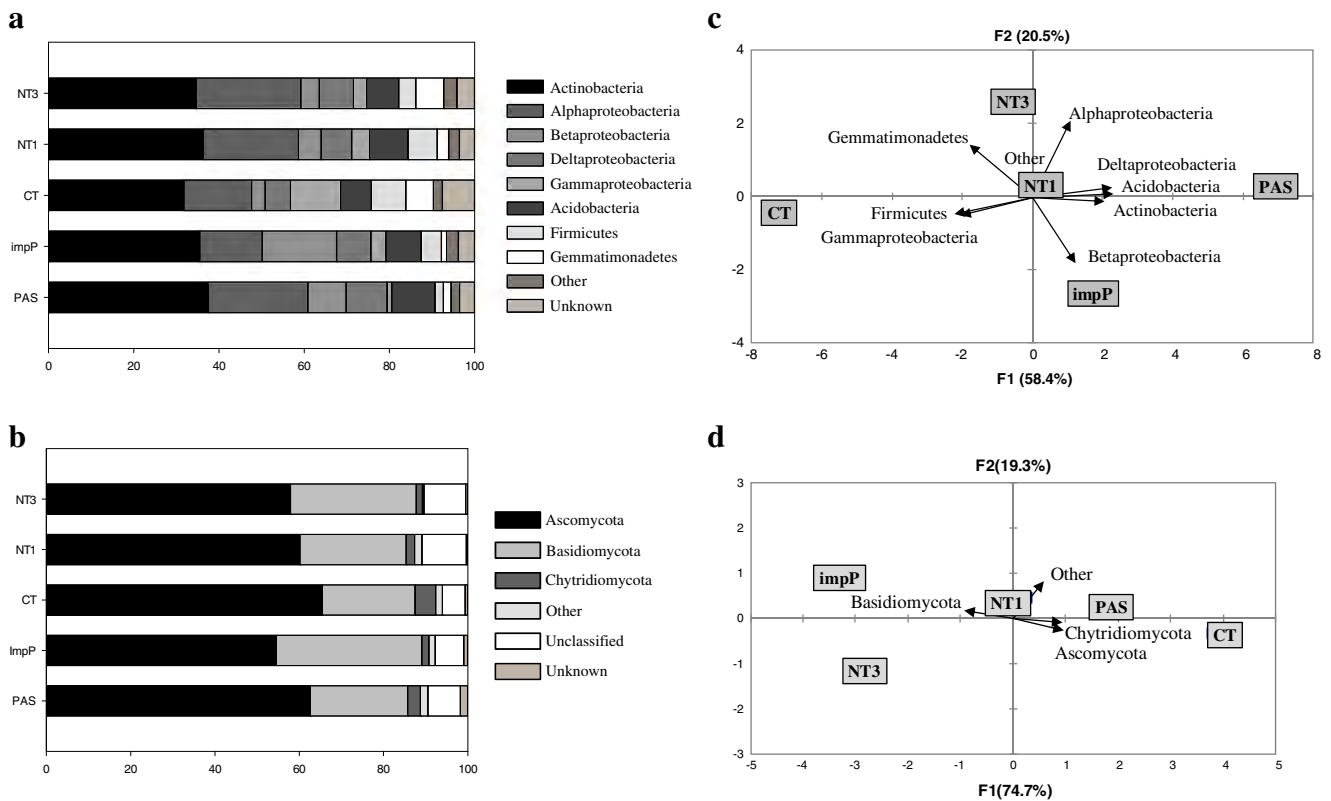


Fig. 3 Bar plot representation of **a** soil bacterial and **b** soil fungal phyla relative abundance according to land use management, and factorial maps of the PCA performed on **c** soil bacterial and **d** soil fungal phyla

soils (Buee et al. 2009; Nishizawa et al. 2010; Yu et al. 2011). However, land use management affected phyla relative abundance distribution, with a higher relative abundance of *Basidiomycota* observed under ruzi grass-dominated systems as compared to the conventional tillage (+12 % and +8 % for improved pasture and no-till system 3, respectively), and by contrast a lower relative abundance of *Ascomycota* (−11 % and −7 %, respectively) and *Chytridiomycota* (−3 % and −3.5 %, respectively) under improved pasture and no-till system 3 as compared to conventional tillage (Fig. 3d). Our results are consistent with de Boer et al.'s (2005) description of *Basidiomycotina* fungi's predominant role in lignin degradation, with a higher amount of stubble restitution under improved pasture and no-till system 3 than under conventional tillage (Lienhard et al. 2013). In addition, soil *Chytridiomycota* have been shown to be able to recover from drying and high temperature events (Gleason et al. 2004), which may more likely occur under bare and tilled soils (Six et al. 2006).

Altogether, the analysis of soil microbial phyla distribution gives us an interesting overview of the ecological status at evaluation through the ecological attributes of the microbial groups that were stimulated. It is worth noting that all the sub-taxa of these broad phyla may clearly not conform to these general ecophysiological characteristics (oligotrophic vs

relative abundance. Natural pasture (*PAS*), improved pasture (*impP*), conventional tillage (*CT*), no-till systems (*NT1* and *NT3*)

copiotrophic attributes). However, our results suggest that general ecological attributes may be ascribed at the phyla level, in agreement with other authors (Cleveland et al. 2007; Fierer et al. 2007; Jenkins et al. 2010).

4 Conclusion

In an acid tropical grassland environment, we observed an early and significant effect of agricultural management on soil microbial properties, with tillage decreasing fungal richness and diversity, but increasing bacterial richness and diversity. We found also that land use modified soil microbial taxonomic composition. Compared to the natural pasture, tillage decreased notably the relative abundance of *Actinobacteria*, *Acidobacteria*, and *Delta-proteobacteria* phyla, and by contrast increased the relative abundance of *Firmicutes*, *Gamma-proteobacteria*, and *Chytridiomycota* phyla. Consequently, our results highlight that no-till cropping systems represented intermediate situations between tillage and the natural pasture, and therefore suggest the promotion of no-till systems as a fair trade-off between the need for agriculture intensification and soil ecological integrity preservation.

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Turnover of soil bacterial diversity driven by wide-scale environmental heterogeneity

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Spatial scaling and determinism of the wide-scale distribution of macroorganism diversity has been largely demonstrated over a century. For microorganisms, and especially for soil bacteria, this fundamental question requires more thorough investigation, as little information has been reported to date. Here by applying the taxa-area relationship to the largest spatially explicit soil sampling available in France (2,085 soils, area covered $\sim 5.3 \times 10^5 \text{ km}^2$) and developing an innovative evaluation of the habitat-area relationship, we show that the turnover rate of bacterial diversity in soils on a wide scale is highly significant and strongly correlated with the turnover rate of soil habitat. As the diversity of micro- and macroorganisms appears to be driven by similar processes (dispersal and selection), maintaining diverse and spatially structured habitats is essential for soil biological patrimony and the resulting ecosystem services.

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Since the first statement of microbial biogeography developed by Baas Becking¹ in 1934 “Everything is everywhere, but, the environment selects”, it is now well established that soil microbial communities exhibit biogeographical patterns, that is, variations in their distribution across space and time². Although the increasing number of studies of spatial microbial ecology from local to wide scale³ is helping to identify ecological processes (selection, dispersal, ecological and evolutionary drifts, speciation) impacting microbial diversity, the hierarchy of these processes remains unclear in comparison with that of macroorganisms.

One way to tackle the spatial processing of microbial diversification is to evaluate the empirical relationship between geographical distance and genetic similarity of microbial assembly^{4,5}, also known as the distance–decay relationship or the taxa–area relationship (TAR)⁶. Application of the TAR has demonstrated that the species richness of eukaryotic macroorganisms increases with the sampling area⁷. This significant rate of diversity turnover has generally been explained by a dynamic equilibrium between extinction and immigration (“area” hypothesis, mediated by dispersal) and/or by the increase of habitat diversity with increasing area (“habitat heterogeneity” hypothesis, mediated by environmental selection)⁷. In microorganisms, phylogeographic studies have revealed that the genetic similarity within particular populations decreases with distance, which suggests a significant influence of dispersal limitation, leading to strong endemism³, and contradicts the hypothesis of microbial cosmopolitanism⁸. At the community level, biogeographical studies have indicated that diversity turnover is influenced both by geographical distance⁹ and environmental heterogeneity (as classified by soil, vegetation and climatic characteristics)^{10,11}. However, few in-depth studies have been carried out on the TAR of soil microorganisms, especially for soil bacteria, leading to a lack of concepts concerning the diversification processes.

TAR was first reported for plants in 1921 by Arrhenius¹² as a power-law relationship:

$$S_A = S_0 A^z, \quad (1)$$

where S_A is the number of taxa recorded in an area A , S_0 the initial number of taxa in the smallest sampling area and z is the rate at which new taxa are sampled as the sampling area is increased. TAR was extended to microorganisms less than a decade ago^{3,9,13} by focusing on community similarity between sites:

$$\chi_d = \chi_D \left(\frac{d}{D} \right)^{-2z}, \quad (2)$$

where z is the turnover rate as in equation (1), χ_d and χ_D are the operational taxonomic unit (OTU_{bin}) similarities between sites located d meters and D meters apart from each other (d^2 and D^2 would correspond to the area of the sampled locations), respectively. Nowadays, TAR is commonly used in this form in most microbial biogeographical studies^{3,9,14–16} to assess microbial diversification and its potential relative dependency on “dispersal” and “habitat heterogeneity” (including habitat diversity and landscape configuration)⁴. Nevertheless, the TAR is still highly debated, particularly regarding its form, underlying hypotheses and factors affecting the relationship^{17–19}. Rosindell *et al.*¹⁹ highlighted that the TAR follows a power-law relationship under the hypothesis of infinite community size and for a steady z . As the soil microbial community is very large and diverse²⁰, the relationship for these organisms exhibits power-law behaviour because the average abundance per microbial taxonomic unit is large according to Harte *et al.*²¹ On the other hand, the constant z

hypothesis across spatial scales, which assumes self-similarity as a probability rule^{13,18} for the spatial distribution of taxa abundance, may not hold for soil microbes^{15,21,22}. Therefore, z may be considered constant only at a given scale of investigation, not across spatial scales²¹, and such spatial variations must be specified for microbial communities.

Now, it is crucial to investigate whether spatial variations of soil bacterial communities observed on a wide scale comply with the Baas Becking postulate¹ involving solely environmental selection as related to habitat-related environmental heterogeneity, or if other ecological processes could be involved in these patterns (for example, dispersal in the context of Neutral Theory). In this context, at least two objectives might be reached, (i) determine the extent to which soil bacterial diversity increases with sampling area by applying TAR from local to wide scales^{6,23}, and (ii) evaluate the link between biodiversity turnover, environmental heterogeneity and dispersal by confronting community and habitat diversity turnover rates.

In this study, we attained these objectives by conducting a wide-scale investigation on a national soil survey: the French Soil Quality Monitoring Network (“Réseau de Mesures de la Qualité des Sols”, RMQS) that covers the huge environmental diversity of whole France (2,085 sites analysed)²⁴. Variations in microbial community between soils were assessed by bacterial Automated Ribosomal RNA Intergenic Spacer Analysis (ARISA) directly on soil DNA²⁵. The TAR was then applied to ARISA data by considering the slope (z_{bacteria}) together with the initial similarity⁵ (χ_{16}) at the grain size of the study (16 km) and the average similarity ($\bar{\chi}$) as proposed by Morlon *et al.*⁴ to complete the analysis of community spatial variations. In parallel, soil habitat, namely the “description of a physical place, at a particular scale of space and time, where an organism either actually or potentially lives”²⁶, was characterized by including pedoclimatic, geomorphologic and land-use data. The TAR concept was then transposed to habitat, assuming that its similarity would decrease with increasing distance (habitat–area relationship (HAR)). For TAR and HAR, the dependence of the slopes and average similarities on spatial scales was evaluated on the scale of France, after which the study of ecological processes was focused on a circular neighbourhood 280 km in diameter. The slopes of the TAR and HAR, as well as the initial similarities and average similarities at this spatial scale, were then confronted and highlighted that dispersal limitation and environmental heterogeneity were not mutually exclusive to determine the bacterial diversity turnover.

Results

Soil-sampling network. The French Soil Quality Monitoring Network represents 2,085 analysed sites, 2,172,570 pairwise comparisons with distances ranging from 16 km to *ca.* 1,100 km and covers more than 550,000 km². Through this network, the enormous environmental heterogeneity of France was examined from a huge number of combinations of soil physico-chemical characteristics, geomorphologic characteristics, land uses and climatic conditions (Table 1 and Fig. 1).

The range of spatial structures for soil bacterial communities and soil habitat characteristics was estimated by computing experimental variograms for each axis of the principle component analysis (PCA) analyses of bacterial community data and habitat data. The results are presented in Table 2. The nugget effects were large for bacterial communities (nugget:sill ratio of 0.57 to 0.85) and smaller for soil habitat (nugget:sill ratio of 0.21 to 0.68). The effective variations for the different axes, both for habitat and soil bacterial community, ranged from 150 to 470 km and highlighted large spatial structures. The size of the spatial structures

Table 1 | Summary statistics of habitat variables.

	<i>n</i>	Mean (\pm s.e.)	Range	CV (%)
Clay (g kg ⁻¹)	2,085	245.2 \pm 2.9	(2–819)	54
Silt (g kg ⁻¹)	2,085	406.2 \pm 3.8	(2–819)	43
Sand (g kg ⁻¹)	2,085	348.6 \pm 5.1	(7–986)	67
pH _{water}	2,085	6.4 \pm 0.03	(3.7–9.2)	21
C _{org} (g kg ⁻¹)	2,085	25.4 \pm 0.4	(0.6–243.0)	80
Total N (g kg ⁻¹)	2,085	2.1 \pm 0.03	(0–16)	73
CaCO ₃ (g kg ⁻¹)	2,085	53.8 \pm 2.9	(0–866)	250
C:N	2,085	12.2 \pm 0.1	(4.6–52.7)	37
Mean annual temperatures (°C)	2,085	11.6 \pm 0.03	(0.2–16.2)	14
Sum of annual rainfall (mm)	2,085	935.3 \pm 4.9	(535.7–2158.2)	24
Annual evapotranspiration (mm)	2,085	811.9 \pm 3.0	(501.6–1560.3)	17
Altitude (m)	2,085	326 \pm 8.8	(0–2980)	124
Slope (%)	2,085	6.6 \pm 0.2	(0–103.2)	159
Orientation (°)	2,085	179.9 \pm 2.4	(–1–359.5)	60
Land uses				
Croplands	869			
Forests	567			
Vineyards	36			
Grasslands	523			
Others	90			

CV, coefficient of variation. Range: (minimum–maximum). Orientation corresponds to that of the largest slope in the grid cell. 'Others' for land uses represents particular land use such as orchards, wild lands, urban parks and specific ecosystems like upland meadows.

explaining the most the inertia of bacteria and habitat variations (PCA axis 1 in Table 2) ranged from 250 to 320 km.

Distance decay of bacterial community and habitat. The TAR measures the spatial turnover of community richness and allows the rate of community composition turnover (commonly referred to as z) to be calculated. According to Green *et al.*⁹, z can be equivalently calculated from the increase of community richness with sampled area or from the decay of similarity in the community composition with increasing distance between sampling sites. Here the bacterial community composition of the soil samples was characterized by ARISA and the samples were sorted into OTUs.

Bacterial community similarity on the scale of France decreased significantly with geographical distance, indicating that the TAR was significant and demonstrating a spatially structured distribution of soil bacteria over a distance ranging from 16 to 1,100 km (Fig. 2a). At this scale, the estimated z_{bacteria} was $0.006 (\pm 7 \times 10^{-4}, P < 0.001)$.

The turnover rate of habitat (z_{habitat}) was determined by transposing the TAR to habitat, assuming that the HAR was a power-law relationship (Fig. 2b). Similarly, a significant HAR was also found on the scale of France with an estimated z_{habitat} of $0.023 (\pm 1 \times 10^{-5}, P < 0.001)$.

Influence of spatial scale on TAR and HAR. As the parameters (initial similarity and turnover rate) of the TAR, and therefore those of HAR by transposition, might be affected by the spatial scale considered, the regression parameters of the TAR and HAR were compared for different spatial scales (0–70, 70–140, 140–280, 280–560 and 560–1120 km, Fig. 3). The z_{bacteria} ranged from 0.003 to 0.010, depending on the scale. The z_{bacteria} was not significantly different at the smallest (0–70 and 70–140 km) and the largest (560–1120 km) scales, but was significantly higher than at the intermediate scales (140–280 and 280–560 km). Conversely, the z_{habitat} presented higher values than the z_{bacteria} (from 0.018 to 0.055) and was positively correlated with spatial scale. Interestingly, z_{habitat} increased moderately and regularly up to the

280–560 km scale, but increased very drastically at scale larger than 580 km.

The average similarities of bacterial communities and habitat ranged from 0.62 and 0.64 to 0.71 and 0.81, respectively. The ranges of variations were small, even if significant differences were observed between every spatial scale with a decreasing trend from small to large scales. The initial similarities of bacterial community (χ_{16}) or habitat (E_{d16}) were defined at the grain size of the study (16 km) and could not be calculated over multiple spatial scales. Nevertheless, this metric was compared with the average similarity at different scales. Here, χ_{16} and E_{d16} were 0.65 and 0.84, respectively, representing higher values than the average similarities calculated at different scales.

Relationship between initial or average similarities. Here the objective was to determine if bacterial similarity was determined by the similarity of habitat at a local spatial scale (16 km) and at the patch scale, that is, if similar habitats house similar bacterial communities. The confrontation of initial similarities (χ_{16} and E_{d16} , Fig. 4a) exhibited a relationship modelled with a linear regression type II model. The associated permutation test highlighted the significance of this relationship (1,000 permutations, $r^2 = 0.123, P < 0.001$). In this relationship, χ_{16} decreased with increasing E_{d16} , but the range of variations of χ_{16} and E_{d16} remained small.

The average similarity of bacterial community structure was significantly correlated to the average habitat similarity ($r^2 = 0.036, P < 0.05, 1,000$ permutations Fig. 4b), but the range of variations of $\bar{\chi}$ and \bar{E}_d remained small.

In addition, χ_{16} and $\bar{\chi}$, and E_{d16} and \bar{E}_d were also correlated ($r = 0.982$ and 0.806 , respectively).

TAR–HAR relationship. The slopes of the TAR and HAR were confronted to evaluate if the turnover rate of soil bacterial community was determined by the turnover rate of soil habitat. To achieve this goal, the statistical confrontation of z_{bacteria} and z_{habitat} was performed by systematically defining TAR and HAR in a neighbourhood corresponding to a circular sliding window of

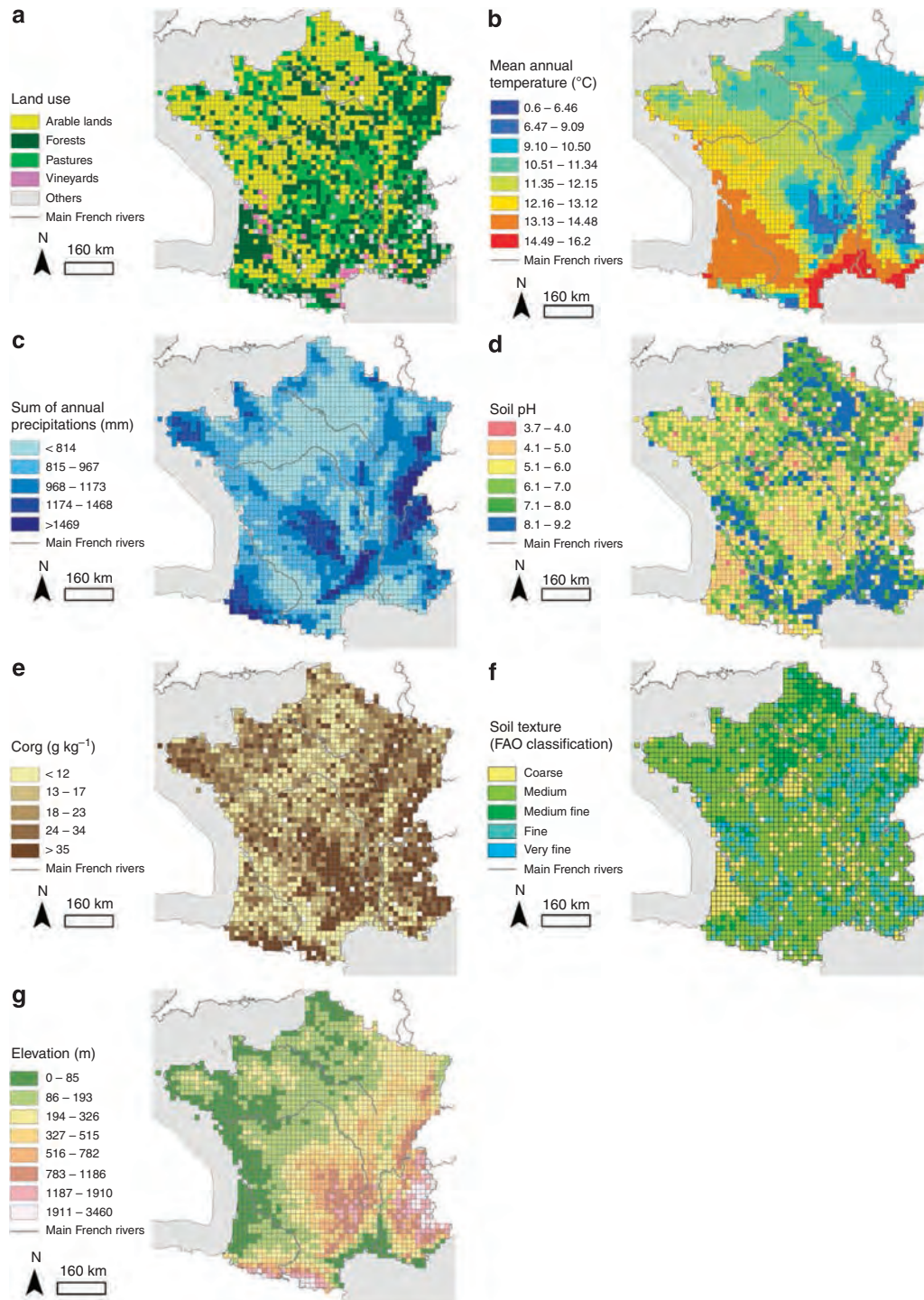


Figure 1 | Environmental variability across France. (a) Map of land-uses according to the Corine land cover classification; (b, c) maps of climatic conditions: mean annual temperature and sum of annual precipitations, respectively; (d-f) Maps of soil physico-chemical characteristics: pH, organic carbon content and soil texture according to the Food and Agriculture Organization (FAO) classification; (g) map of elevation (m).

280 km diameter, centred on each of the 2,085 RMQS sites. The window size was deduced from the size of spatial structures of bacteria and habitat identified by the experimental variograms on the scale of France (Table 2)²⁷.

The range of variations in z_{bacteria} and z_{habitat} could thus be determined and showed that z_{bacteria} ranged from -0.003 to $+0.021$ (mean = 0.008, median = 0.009), whereas z_{habitat} ranged from -0.002 to 0.131. For z_{bacteria} , the negative turnover rates

(<1% of total estimates) were observed in some regions where distant sites were more similar to those in the centre, than to intermediate sites. Although in 62% of the regions defined by the sliding windows, the TAR ranged from 0.002 to 0.01 and was close to that observed for France as a whole, in 36% of the regions, the TAR was significantly higher and ranged from 0.01 to 0.02. In other respects, the value of z_{habitat} calculated for France (0.023) was lower than the mean (0.040) or median (0.035)

obtained for the 2,085 regions. In some regions (<1%), z_{habitat} was negative because distant sites showed greater similarity to those in the centre than to intermediate sites.

Statistical confrontation of the 2,085 z_{bacteria} and z_{habitat} estimates revealed a linear relationship (Fig. 5). The significance of the relationship was tested through a linear regression type II model²⁸, as both estimated turnover rates were associated with errors. A permutation test (random association of z_{habitat} and z_{bacteria} values, 1,000 permutations)²⁹ showed that the model and the regression parameters were highly significant ($r^2 = 0.667$, $P < 0.001$ and Fig. 5).

Influence of landscape configuration. Habitat turnover rate and initial similarity are determined by habitat diversity and its spatial configuration (that is, landscape configuration). In the previous section, the permutation test demonstrated that habitat diversity significantly influenced the relationship between habitat and bacterial community turnover and initial similarities. Therefore, in this section the objective was to evaluate the importance of landscape configuration on these relationships. To achieve this goal, a permutation test was performed in which the spatial organization of habitat was reconfigured without modifying the bacterial community variability, habitat variability or the selection pressure of habitat on the bacterial community (pairs of habitat and bacterial community composition data unmodified). The

modification of landscape configuration significantly altered the correlation (Fig. 6a, b) between z_{bacteria} and z_{habitat} , and changed the sign of the correlation between χ_{16} and E_{d16} in the random case. This might be related to the range of variations of χ_{16} , which was smaller than that of E_{d16} . Similarly, the intercepts of the relationships β_z and β_z were significantly reduced by landscape reconfiguration: 0.004 versus 0 and 0.820 versus 0.558, respectively (Fig. 6c, d). The slope of the relationship between the initial similarities (α_z) was also affected by landscape reconfiguration (-0.221 versus 0.108 in the random case Fig. 6f), but this was not the case for the slope α_z that harboured very similar values for the observed and the random cases (0.1350 and 0.1231 , respectively, Fig. 6e).

Discussion

In this study, a significant turnover of soil bacterial diversity was demonstrated on a broad scale. This is an important and relevant finding, as several aspects of bacterial physiology and ecology (ubiquity, horizontal gene transfer, high ability to colonize extreme environments, redundancy of ecological attributes)²⁰ would be expected to prevent a significant TAR for these organisms. Significant TAR have already been reported for indigenous bacteria from various ecosystems, such as water-filled tree holes³⁰ or salt marshes¹⁴, which suggests that TAR is a universal relationship for all living organisms and all environments.

The estimated z_{bacteria} was $0.006 (\pm 7 \times 10^{-4})$, $P < 0.001$, that is, within the lower range of turnover rates for microbes ($0.002 < z < 0.26$), as compiled by Woodcock *et al.*³¹ This is in agreement with the general trend observed for microbes, the values usually being well below than those of the macroorganisms ($z_{\text{macroorganisms}} \geq 0.1$)¹⁴, even if a higher z_{bacteria} (0.26) has been reported for small, discrete and highly heterogeneous ecosystems³⁰ known as hosting insular communities with a high community turnover³². The significant but low turnover observed on a wide scale in our study might partly be ascribed to the high average abundance per taxonomic unit, which induces low turnover rates²¹, and to technical limitations, particularly the low taxonomic resolution of DNA fingerprinting. Indeed, DNA fingerprints correspond to dominant bands, each including multiple species. This results in an aggregation of species composition variations within the bands and may contribute to the low turnover rate observed⁴. In addition, DNA fingerprinting precludes consideration of the accumulation of new minor species

Table 2 | Fitted parameters of the variograms of bacterial genetic structure and soil habitat.

Data	PCA axis number	Inertia (%)	S = nugget:sill ratio	Effective range (km)
Bacterial community structure	1	22.6	0.57	249.6
	2	10.7	0.79	166.4
	3	6.9	0.85	416.1
	4	6.4	0.78	249.6
	5	5.7	0.78	249.6
Soil habitat	1	21.4	0.21	323.4
	2	17.7	0.46	154.7
	3	11.2	0.43	421.8
	4	7.3	0.68	469.5
	5	6.5	0.67	295.3

PCA, principle component analysis. The different parameters were computed for each axis of the PCAs performed on genetic and environmental data, respectively.

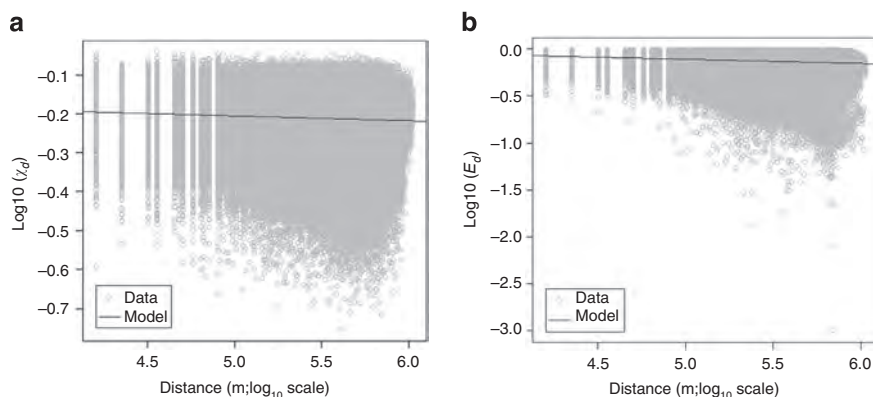


Figure 2 | TAR and HAR relationships at the scale of France. (a) TAR, (b) HAR, grey circles: logarithm of genetic and habitat similarity values for each paired comparison of sites confronted with the logarithm of between-sites geographical distance (m); black line: weighted linear regression model for the distance-decay relationships. (a) Significant model ($P < 0.001$): $\log_{10}(\text{genetic similarity}) = -0.0117 \cdot \log_{10}(\text{geographical distance}) - 0.1464$. (b) Significant model ($P < 0.001$): $\log_{10}(\text{habitat similarity}) = -0.0454 \cdot \log_{10}(\text{geographical distance}) + 0.1174$.

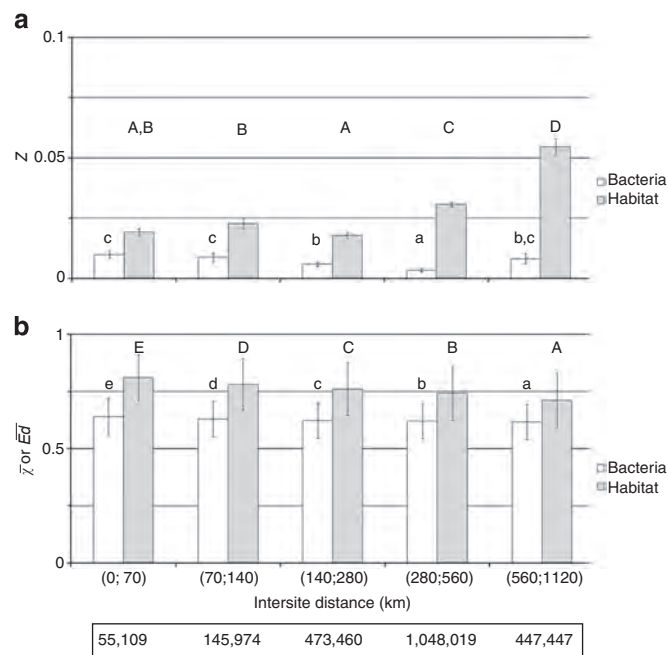


Figure 3 | Scale effects on TAR and HAR parameters. Variations of z_{bacteria} and z_{habitat} (a), and of the average similarity of bacterial community (\bar{z}) or habitat (\bar{E}_d) (b) at different spatial scales. Error bars correspond to 95% intervals for regression parameters (z) and to s.d. for average similarity of bacterial community or habitat. The result of the statistical comparisons of z or average similarities between spatial scales is indicated through capital letters for habitat and through lower case letters for bacteria. The box at the bottom of the figure indicates the number of pairwise comparisons per spatial scale.

with increasing distance, which may represent a large proportion of the total soil microbial species richness³³ and determine the slope of TAR^{14,31}. The low z_{bacteria} might also be related to the grain size of our sampling design (16 km × 16 km: 256 km²), which did not consider scales less than landscape, and smoothed significant local variations in soil microbial community composition that might potentially affect TAR³⁴.

In parallel, soil habitat was characterized by a multivariate analysis including pedoclimatic, geomorphologic and land-use data, and the HAR was calculated by transposing the TAR concept. On the scale of France, z_{habitat} was 0.023 ($\pm 1 \times 10^{-5}$, $P < 0.001$). This value can only be considered in relation to z_{bacteria} , as no other means of comparison is available in the literature. The z_{habitat} was higher than the z_{bacteria} , which might be related to the lower average abundance per habitat than per bacterial OTU_{bin}.

As described in previous studies, TAR is sensitive to the spatial scale of investigation²¹. The sampling design applied in our study allowed us to test this hypothesis on both TAR and HAR, and to consider multiple spatial scales (distance range between site: [0; 70], [70–140], [140; 280], [280; 560] and [560; 1100] km). The significant effect of spatial scale on the average similarities of soil bacterial community and habitat and on the turnover rates was demonstrated. The average similarities decreased significantly with increasing spatial scale and were always lower than the corresponding initial similarity (0.65 and 0.84 for χ_{16} and E_{d16} , respectively). This is in agreement with the hypothesis that neighbouring sites are more similar than distant sites^{2,15}. The significant decrease of z_{bacteria} with increasing spatial scales might be explained by the higher average abundance per OTU with increasing scales^{21,22}. Similarly, the significant increase of z_{habitat}

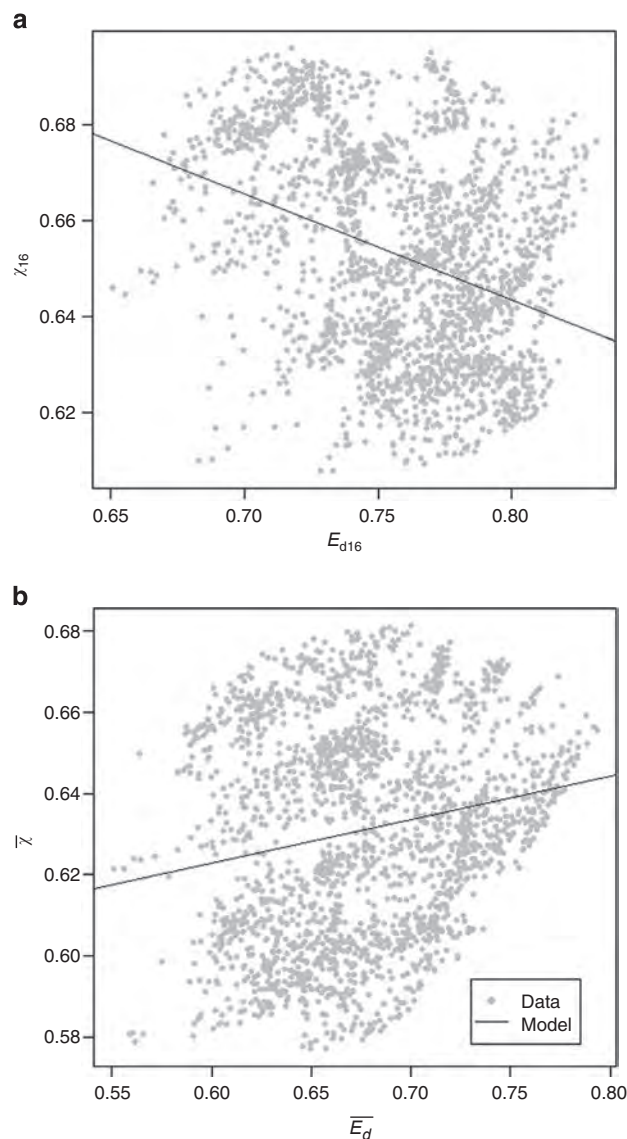


Figure 4 | Relationship between bacterial communities and habitat similarities. (a) Initial similarities at 16 km; and (b) mean similarity of habitat and bacterial community estimated for each RMQS site considering every site within a circular neighbourhood with a 280 km in diameter. Black line: best significant model fitted to the data ($P < 0.001$, 1,000 permutations): (a) $\log_{10}(\chi_{16}) = -0.2210 \cdot \log_{10}(E_{d16}) + 0.8204$, (b) $\bar{z} = 0.1071 \cdot \bar{E}_d + 0.5586$.

with spatial scale might result from decreasing average abundance per habitat, as the number of observations per habitat increases equally or even more slowly than habitat richness.

Altogether, the significant variations of z_{bacteria} and z_{habitat} according to scale might reflect variations in the relative importance of diversification processes⁴. Morlon *et al.*⁴ demonstrated the relevance of distance–decay relationships for testing spatial ecology theories. We therefore compared three metrics relating bacterial community and habitat, namely turnover rates, initial and average similarities, to identify the processes involved in the spatial distribution of soil bacterial communities. These comparisons were performed by computing and compiling data from the 2,085 circular neighbourhoods (280 km in diameter as determined from experimental variograms, see Table 2). At this spatial scale, the main

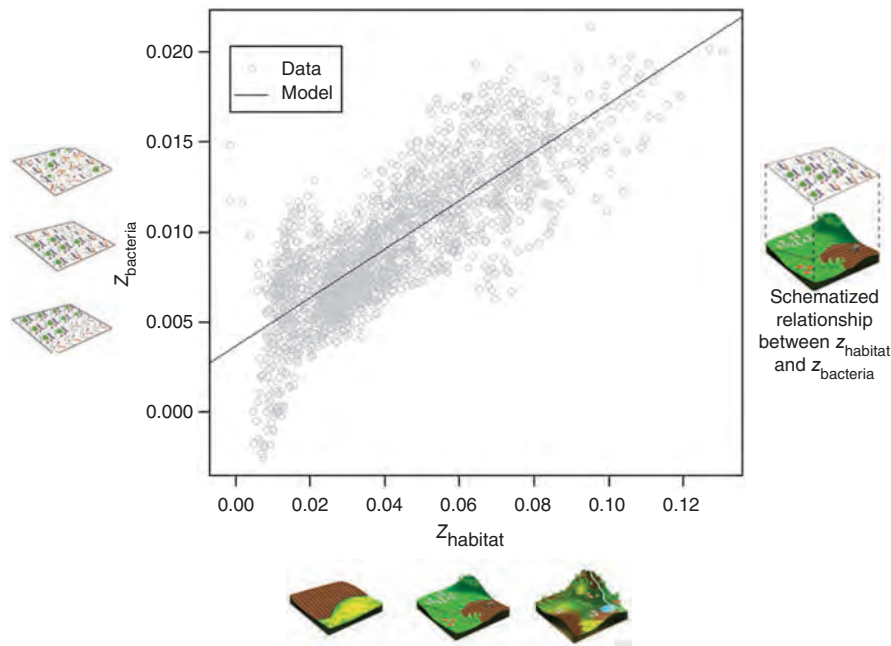


Figure 5 | Relating turnover rates of soil bacterial community structure and habitat. Grey circles represent the turnover rates calculated for each RMQS site considering every site within a distance of 140 km in a circular window. The black line represents the linear model type II fitted to the data: $z_{\text{bacteria}} = 0.1350 * z_{\text{habitat}} + 0.0036$, ($r^2 = 0.65$, $P < 0.001$). Representation of diversity and spatial fragmentation of soil bacterial community assemblage and of habitats are schematized by the y axis and under the x axis, respectively.

processes to consider were (i) environmental heterogeneity leading to adaptation of populations to a particular habitat, the larger areas exhibiting a higher renewal of habitat variation^{2,30}, that is, the “environmental selection hypothesis”, and (ii) “dispersal limitations” resulting from the balance between species extinction and colonization, which is significantly influenced by the size of the sampling area. If dispersal is infinite, community composition is fully determined by environmental selection, bearing out the postulate of Baas Becking¹: “Everything is everywhere, but, the environment selects”. Under this postulate, the turnover rate of the soil bacterial community would be entirely proportional to the turnover of the soil environment and would, on average, be zero in completely homogeneous soil environments ($z_{\text{habitat}} = 0$). Otherwise, if the dispersal of soil bacteria is limited, significant turnover rates of bacterial community would be observed in homogeneous habitats according to the Neutral Theory^{19,35}. Under infinite dispersal, the same interpretation would hold for the relationships between the initial or average similarities of soil bacterial community and habitat, which should be positive and reach the maximum in fully homogeneous habitat.

Here the average habitat similarity and average bacterial community similarity were significantly and positively correlated at the scale of the circular neighbourhoods, which was in agreement with the environmental selection hypothesis^{2,16,36}. Nevertheless, the initial similarities of habitat and soil bacterial community were weakly but negatively correlated, suggesting that variations in community composition occur at small scales, even in homogeneous habitats.

The significant linear regression between z_{habitat} and z_{bacteria} demonstrated a positive relationship between soil bacterial community turnover and habitat turnover on a regional scale (Fig. 5), thereby supporting the environmental selection hypothesis for soil microorganisms^{3,7}. It is interesting to note that z_{bacteria} values may attain those reported for larger organisms (*ca.* 0.02), even in large areas of contiguous habitats, suggesting that

communities of both bacteria and macroorganisms are structured by analogous processes³¹. Recent microbial biogeography studies have revealed that the main environmental filters shaping spatial microbial diversity distribution are soil physico-chemical characteristics, land use and plant cover, whereas climatic and geomorphologic filters are less important^{9,11,15,37}. Similar filters were also reported to explain soil bacterial community distribution in contrasted ecological regions at the scale of France^{27,34}.

Nevertheless, the estimated z_{bacteria} in the case of a homogeneous habitat ($z_{\text{habitat}} = 0$) was not equal to 0, suggesting that bacterial communities might have a limited dispersion despite the huge amounts of bacteria ($> 10^{15}$ cells per year) transported through the atmosphere between continents³⁸. This conclusion for the soil bacterial community as a whole is in agreement with phylogeographic studies focusing on particular soil bacterial groups^{15,16,39} and with studies showing a distance effect on soil bacterial community structure².

We then determined the relative dependency of bacterial community turnover on dispersal limitation by testing the effect of habitat spatial configuration on the relationships between z_{bacteria} and z_{habitat} , and between the initial similarities (χ_{16} and E_{d16}) without modifying the environmental selection process, that is, the pairs of soil habitat characteristics and associated bacterial community composition data. By shuffling habitat spatial configuration, we altered the relationships between turnover rates and between initial similarities of bacterial community and habitat, respectively (Fig. 6). The versatility of the correlation (in terms of sign and significance) between the initial similarities confirmed the difficulty of drawing conclusions at the grain size of this study. On the other hand, the relationship between turnover rates was significantly altered for the correlation and intercept, but not for the slope. This supported the hypothesis that the slope of the relationship between turnover rates is determined by the selection pressure of the soil habitat on the indigenous bacterial community. It also suggested that the intercept of the relationship (bacterial diversity turnover rate in

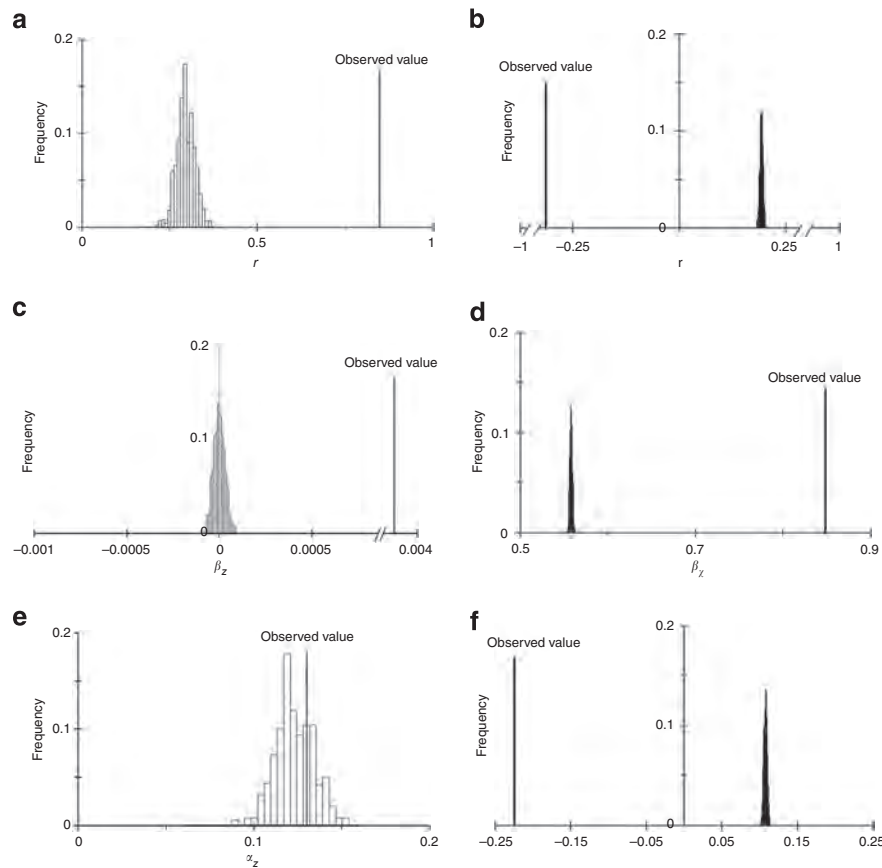


Figure 6 | Effect of landscape on microbial and habitat turnover rates or initial similarities. (a) Distribution of the correlation coefficient (r) of the relationship between turnover rates; (b) distribution of the correlation coefficient (r) of the relationship between initial similarities; (c) distribution of the intercept (β_z) of the relationship between turnover rates; (d) distribution of the intercept (β_z) of the relationship between initial similarities; (e) distribution of the slope (α_z) of the relationship between turnover rates; (f) distribution of the slope (α_z) of the relationship between initial similarities. Each distribution is based on 500 permutations in each sliding window.

homogeneous habitats) is related to dispersal limitations mediated by the spatial configuration of the habitat. This statement would be in favour to the role of population aggregation in determining the turnover rate of bacterial diversity⁴.

Our study negates the hypothesis that the soil microbial community is a “black box” with no spatial structure and exhibiting a homogeneous distribution from local to large scales. As observed in macroorganisms, spatial patterns in soil bacterial communities are also significantly governed by environmental heterogeneity and dispersal, and the two processes are not mutually exclusive^{2,7}. Moreover, the environmental filters impacting the turnover of soil microbial diversity as a whole need to be deciphered and ranked to better understand the sustainability of the soil ecosystem services provided by the corresponding biodiversity. This represents a major challenge in microbial ecology, but should provide the knowledge required for sustainable soil management and implementation of the corresponding protection policies on a wide scale⁴⁰.

Methods

Soil-sampling strategy. The soil samples were provided by the platform GenoSol (http://www.dijon.inra.fr/plateforme_genosol) from the soil storage facility of the RMQS (French Monitoring Network for Soil Quality), which is a soil-sampling network based on a 16 km × 16 km systematic grid covering the whole of France⁴¹. Each of the 2,195 monitoring sites has been precisely geopositioned, and the soil profile, site environment, climatic factors, vegetation and land use have been accurately described. In the centre of each 16 km × 16 km square, 25 individual core samples were taken from the topsoil (0–30 cm) within a 20 m × 20 m area, using an unaligned sampling design. The core

samples were then bulked to obtain a composite sample for each site. The soil samples were gently air-dried, sieved to 2 mm and stored at $-40\text{ }^{\circ}\text{C}$ before DNA analysis.

Physical and chemical analyses (listed in Table 1 and Fig. 1) are available for 2,131 soils and were performed by the Soil Analysis Laboratory of INRA (Arras, France, <http://www.lille.inra.fr/las>). Available climatic data were monthly rain, Potential EvapoTranspiration and temperature at each node of a 12 km × 12 km grid, averaged for the 1992–2004 period. These climatic data were obtained by interpolating observational data using the SAFRAN model⁴². The RMQS site-specific data were linked to the climatic data by finding the closest node from the 12 km × 12 km climatic grid for each RMQS site. Land cover was recorded according to the CORINE Land Cover classification at level 1 (<http://www.ifen.fr>), which consists of a rough descriptive classification of land use into five classes: arable lands, forests, pastures, vineyards and other land uses. All these data were available for 2,085 soils in the INRA InfoSol DONESOL database (http://www.gissol.fr/programme/rmqqs/RMQS_manuel_31032006.pdf; Table 1 and Fig. 1).

Characterization of habitat. Soil habitat was characterized for each site by its coordinates in a Hill and Smith multivariate analysis⁴³ using the `dudi.hillsmith` function in the `ade4` package⁴⁴ in R, which allows mixing quantitative (geomorphological data: altitude, slope and orientation; climatic data: mean annual temperature, annual rainfall and annual potential evapotranspiration; soil physico-chemical data: clay, silt and sand contents, pH_{water} , organic C content, total N content, CaCO_3 content and C:N ratio) and qualitative variables (land use, five classes; Table 1 and Fig. 1). Quantitative variables were centred and scaled, and the qualitative variables were converted into binary variables. All the quantitative variables had a weight of 1 in the analysis and the binary variables had a weight equal to $1/n$, n being the number of classes observed in the qualitative variables they were derived from.

According to the decrease of inertia per axis, five axes representing 63.9% of the variance in the environmental data were retained to characterize the habitat of each

study site. The coordinates of the sites in the Hill and Smith multivariate analysis were used in the following and referred to as habitat characteristics.

Molecular characterization of bacterial community structure. DNA was extracted from 2,085 soils from the RMQS grid, using a single procedure optimized by Ranjard *et al.*²⁵, which is reliable and robust for the routine analyses of several hundreds of different soils. Soil bacterial community structure was genotyped directly from soil DNA extracts using a bacterial ARISA, fingerprinting approach optimized for medium throughput in the GenoSol platform.

Within the ARISA method, the bacterial intergenic spacers were amplified with the primer set: S-D-Bact-1522-b-S-20/ L-D-Bact-132-a-A-18, with 50 ng of DNA as template, the PCR conditions being described by Ranjard *et al.*²⁵ The S-D-Bact-1522-b-S-20 primer was labelled at its 5' end with the IRD800 dye fluorochrome (MWG SA Biotech, Ebersberg, Deutschland) to allow detection of the PCR fragments by the LiCor DNA sequencer system (ScienceTec, Les Ulis, France). PCR fragments were resolved on 3.7% polyacrylamide gels run under denaturing conditions for 15 h at 3,000 V/60 W on a LiCor DNA sequencer (ScienceTec). The data were analyzed using the 1D-Scan software (ScienceTec). This software converts fluorescence data into electrophoregrams where the peaks represent the PCR fragments. The height of the peaks was calculated in conjunction with the median filter option and the Gaussian integration in 1D-Scan, and represented the relative proportions of fragments in the total products. Lengths (in base pairs) were calculated using a size standard with bands ranging from 200 to 1,206 bp.

Data obtained from the 1D-Scan software (Sciencetec) were converted into a table summarizing band presence (that is, peaks) and intensity (that is, height or area of peak) using the PrepRISA program²⁵. As described in a previous study, 100 peaks, 2 bp resolution and Gaussian peak area were used to provide a robust analysis of bacterial communities²⁵. The resulting bacterial-ARISA data matrix (bacterial communities as rows and bands as columns), takes into account the presence/absence and relative intensity of bands.

Bacterial community and habitat experimental variograms. We used the classical geostatistics approach⁴⁵ to characterize the spatial process of bacterial genetic structure and soil habitat. Exponential variograms have been fitted using weighted least squares on the different experimental variograms computed for each axis of the PCAs performed on genetic and environmental data. In this method, the weights of each lag are the experimental variogram of the lag divided by the number of observation pairs within a bin centred on the lag. To characterize the spatial process, we have calculated first the nugget:sill ratio, which represents the non-explanatory part of the spatial model. Second, we retained also the effective range, which is the distance data that the semivariance value achieves 95% of its maximum.

TAR and HAR computing. The turnover rate of bacterial community composition (z_{bacteria}) was determined by converting molecular fingerprint data into presence/absence data and computing between-sites similarity using the dsdvs function (Sørensen index) of the labdsv package⁴⁶ in the R software⁴⁷. Then, z_{bacteria} was estimated from the slope of the decay relationship between similarity and distance according to the formula:

$$\log_{10}(z_d) = (-2z) * \log_{10}(d) + b \quad (3)$$

where z_d is the observed Sørensen's similarity between two soil samples (number of common OTUs divided by the average number of OTUs in the two samples), which are d meters apart from each other, b is the intercept of the linear relationship and z is the turnover rate of the community composition (referred to as z_{bacteria}). Regression parameters $(-2z)$ and b were estimated by weighted linear regression using the ordinary least squares method. The weight of each similarity value corresponded to the number of data in the respective distance class. This approach was applied to avoid bias related to large distance classes, including few but very dissimilar sites. The significance threshold was set at $\alpha = 0.05$.

The turnover rate of habitat (z_{habitat}) was determined by transposing the TAR to habitat, assuming that the HAR was a power-law relationship. For this purpose, habitat characteristics were used to compute between sites similarity (E_d) as follows:

$$E_d = \left(1 - \frac{Euc_d}{Euc_{\max}}\right) + 0.001 \quad (4)$$

with Euc_d the Euclidean distance (dist function, R software) between two sites that are d meters apart from each other, and Euc_{\max} is the maximum Euclidean distance in the distance matrix; 0.001 was added to account for zero similarity between sites²⁹. Then, z_{habitat} was estimated from the slope of the decay relationship between habitat similarity and distance according to equation (3). Regression parameters were calculated by adopting the same methodology used to calculate the parameters for the TAR.

Average and initial similarities. The average similarity of soil habitat (\bar{E}_d) or of bacterial community composition (\bar{z}) was determined as the mean of the similarity matrix computed for soil habitat or bacterial community as described above.

The initial similarities of soil habitat (E_{d0}) or of bacterial community composition (z_0) were computed as the mean similarity between sites for a distance between sites of 16 km.

Relating habitat and bacterial similarities or turnovers. To evaluate these relationships, a neighbourhood statistics approach was used allowing computation of z_{bacteria} , z_{habitat} , E_{d16} , χ_{16} , \bar{E}_d and \bar{z} for each site in the RMQS on a circular neighbourhood 280 km in diameter. For that purpose, the same methods as those presented in the sections devoted to computing the TAR, the HAR, the average and the initial similarities of soil habitat and bacterial community composition were applied.

The size and shape of the neighbourhood was in agreement with previous spatial analyses of habitat and genetic data that demonstrated significant spatial structures²⁷ when the radius was at least 140 km (Table 2). As all the parameters are estimated with errors, the relationships were best modelled by a type II linear regression model (ordinary least squares method). This was done using the 'lmodel2' function of the 'lmodel2' package²⁸ in the R software. A permutation test (1,000 permutations) was used to test the significance of the relationship and its parameters²⁸. The slope and the intercept were referred as follows: α_z , β_z , α_{z_2} , β_{z_2} , α_z , β_z for the relationship between z_{bacteria} and z_{habitat} , χ_{16} and E_{d16} , $\bar{\chi}$ and \bar{E}_d ; respectively.

Influence of landscape configuration. To evaluate the influence of landscape on the relationship between habitat and bacterial community turnover or between initial similarities, a permutation test (500 permutations) was performed. In this test, the neighbourhood statistics approach described above was used, but the geographic coordinates of the sites in the neighbourhood region (280 km diameter) were shuffled without modifying the pairs of soil habitat characteristics and bacterial community composition data. This allowed creating randomly configured regions in which bacterial community variability (average similarity and variance), habitat variability (average similarity and variance) and selection pressure of habitat on bacterial community were kept unchanged (pairs of habitat and bacterial community composition data unmodified). As a consequence, only turnover rates and initial similarities were affected, but not the average similarities. Then, the relationships between turnover rates or between initial similarities were computed for each permutation. As a result, the distributions of the Pearson's correlation coefficient and of the regression parameters (α_z , β_z , α_{z_2} , β_{z_2}) were considered for each relationship. Then, the probability of observed value were derived from these distributions and confronted to the 5% significance threshold.

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Author contributions

L.R. managed the mathematical strategy and wrote the manuscript with N.C.P.B. N.C.P.B., S.D., J.T. and N.P.A.S. developed the strategy for mathematical data analysis and for the different ecological hypotheses. M.L. contributed to the molecular characterization of bacterial communities. P.A.M. participated to the development of the project and provided insightful comments and assistance in the redaction of the manuscript. F.E.R.M. developed and managed the MicroSol database required to organize and analyze the environmental and microbial metadata. C.J. coordinated the sampling campaigns on the French territory and managed the associated soil archive and data library. A.B., D.A. and P.L. permitted the scientific link between the RMQS sampling strategy and molecular microbial ecology by initiating scientific and technical collaborations.

Additional information

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No-till and cover crops shift soil microbial abundance and diversity in Laos tropical grasslands

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Abstract Agricultural practices affect the physical and chemical characteristics of the soil, which in turn may influence soil microorganisms with consequences on soil biological functioning. However, there is little knowledge on the interactions between agricultural management, soil physicochemical properties, and soil microbial communities, notably in tropical ecosystems with few studies conducted in strongly weathered and acid soils. Here, we investigated the early effect of tillage and crop residues management on top soil physical, chemical, and microbial properties in an acid savannah grassland of northeastern Laos. We initiated a 3-year rotation of rice/corn/soybean under three no-till systems (NTs) distinguished by the cover crops associated prior to and with the main crops, and

one conventional tillage-based system (CT). The effect of agricultural management was evaluated 2 years after land reclamation in reference to the surrounding natural pasture (PAS). Our results demonstrate that NTs improve soil physicochemical characteristics (aggregate stability, organic carbon, and cation exchange capacity) as well as microbial abundance (total biomass, bacterial and fungal densities). A significant discrimination of the genetic structure of soil bacterial community was also observed between NTs, CT, and PAS. Interestingly, bacterial abundance and diversity were differently influenced by soil environment changes: microbial density was affected by the quantity and diversity of crop residues, soil organic carbon, and exchangeable base contents, whereas soil bacterial genetic structure was mainly determined by exchangeable aluminum content, pH, cation exchange capacity, and C/N ratio. Altogether, our study represents one of the most complete environmental evaluations of agricultural practices in tropical agrosystems and leads to recommend no-till systems with high residue restitutions to improve the physical, chemical, and microbial properties of tropical acid soils and thus contribute to the sustainability of agriculture in these ecosystems.

Keywords Tropical soil · Microbial community · Tillage · Conservation agriculture · Cover crop

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1 Introduction

Soil microbial communities are responsible for a wide range of soil functions and ecological services, such as soil structure maintenance, organic matter turnover, and nutrient cycling (Dick 1992; Klavivko 2001). Among human activities, agricultural practices affect the physical and chemical

characteristics of the soil in which microorganisms live, thereby affecting their abundance, diversity, and activity (Dick 1992; Kladivko 2001; Bunemann et al. 2006; Nicolardot et al. 2007; Pascault et al. 2010). External agricultural inputs such as organic amendments (animal or green manure), mineral fertilizers, and pesticides affect in different ways soil microorganisms (Dick 1992; Bunemann et al. 2006). Crop rotation and plant diversity are also important to maintain soil microbial diversity and activity (Nicolardot et al. 2007; Pascault et al. 2010). In conventional agriculture, tillage has generally the greatest impact on biological properties since physical disturbance changes soil water content, temperature, aeration, and the degree of mixing of crop residues within the soil matrix (Dick 1992; Buckley and Schmidt 2001; Kladivko 2001). Tillage also reduces soil macroaggregate content which provides an important microhabitat for microbial density, diversity, and activity (Ranjard and Richaume 2001; Six et al. 2002). In addition, tilling tools disturb fungal hyphal growth at soil surface leading to a reduction of their relative abundance in the soil (Frey et al. 1999; Balesdent et al. 2000).

Based on the principle of minimal soil disturbance, no-till (NT) farming systems have been widely adopted in large-scale mechanized agriculture to prevent soil erosion and decrease production costs (Derpsch et al. 2010). Combined with maximal soil cover (mulch) and diversified crop sequences, NT systems have demonstrated in addition to have a positive impact on soil physical and chemical properties (Castro Filho et al. 2002; Six et al. 2002; Séguy et al. 2006; Lal 2008), and on soil microbial biomass and activity (Kladivko 2001; Kaschuk et al. 2010; Sapkota et al. 2011). However, the adoption of no-till systems in small-scale agriculture is still low (Derpsch et al. 2010) with therefore little data available regarding their environmental impact on soils, notably in tropical grassland ecosystems, whereas the expansion of agriculture is a key challenge in these areas to increase and sustain food production (Lal 2008). If the acid savannah grasslands of the world encompass vast areas of potentially arable land, they are however mostly considered marginal because of low inherent fertility and susceptibility to rapid degradation (IAEA 2000).

In addition, little is known about the impact of agricultural practices on soil microbial communities in tropical agrosystems, and their role in soil biological functioning. The recent development of culture-independent molecular tools based on soil DNA extraction and characterization and of *in silico* meta-analysis have enabled the systematic analysis of soil microbiota leading to a better understanding of the ecological impact of land use management (Maron et al. 2011). Despite these advances, the links between microbial communities and soil physicochemical properties as affected by agricultural practices are still a major challenge and especially in tropical ecosystems where no-till farming systems become an innovative and recurrent way of crop production.

In this context, our objective was to investigate the early effect of tillage systems and crop residue management on top soil physical, chemical, and microbial properties in an acid tropical grassland ecosystem located in the western part of Xieng Khouang province, northeastern Laos. For this purpose, we initiated in 2008 a 3-year rotation of rice (*Oryza sativa* L.), corn (*Zea mays* L.), and soybean (*Glycine max* (L.) (Merr.)) conducted under three no-till systems (NTs) distinguished by the cover crops associated prior to and with the main crops, and one conventional tillage-based system (CT) based on soil plowing with disks and on the burying of crop residues. The impact of agricultural systems was evaluated 2 years after land reclamation in reference to the surrounding natural pasture (PAS). Molecular tools such as soil DNA concentration and real-time quantitative PCR of bacteria and fungi were used as bioindicators to evaluate the effect of agricultural practices on soil microbial abundance (Dequiedt et al. 2011; Smith and Osborn 2009; Chemidlin Prevost-Boure et al. 2011). The genotyping of the soil bacterial community structure was assessed by a DNA fingerprinting approach, the automated ribosomal intergenic spacer analysis technique, that has been demonstrated to be sensitive and relevant for evaluating modifications in microbial community composition consecutive to land use management changes (Pascault et al. 2010; Lejon et al. 2007). The relationships between the soil physicochemical properties and the abundance and diversity of soil microbial communities were statistically tested to deduce an early environmental evaluation of these cropping systems.

2 Material and methods

2.1 Experimental site

The study was conducted in Poa village (Lat. 19°33'N, Long. 102°59' E) at 1,130 m AMSL. The climate is both tropical and mountainous with a 6-month (April–September) wet and hot season and a 6-month dry season including 3 months of cold (December–February). The mean annual precipitation is 1,400 mm. The soils at the site are red Oxisols with clay content decreasing gradually from the upper part (clayey soils, USDA classification) to the lower part (sandy–loamy soils) of the site. The 3-year rotation was conducted in a split–split plot experimental design combining three factors (Fig. 1) with three replications of 270 m² each for a total of 108 sub–sub–plots.

For the present study, we limited our sampling to the upper part of the site (clay dominant soils, total of 12 independent replicates per tillage system, in white font in Fig. 1) since previous studies have shown soil texture as a major factor in explaining soil microbial diversity distribution (Martiny et al. 2006; Dequiedt et al. 2011). We thus decided to minimize the influence of this factor in order to

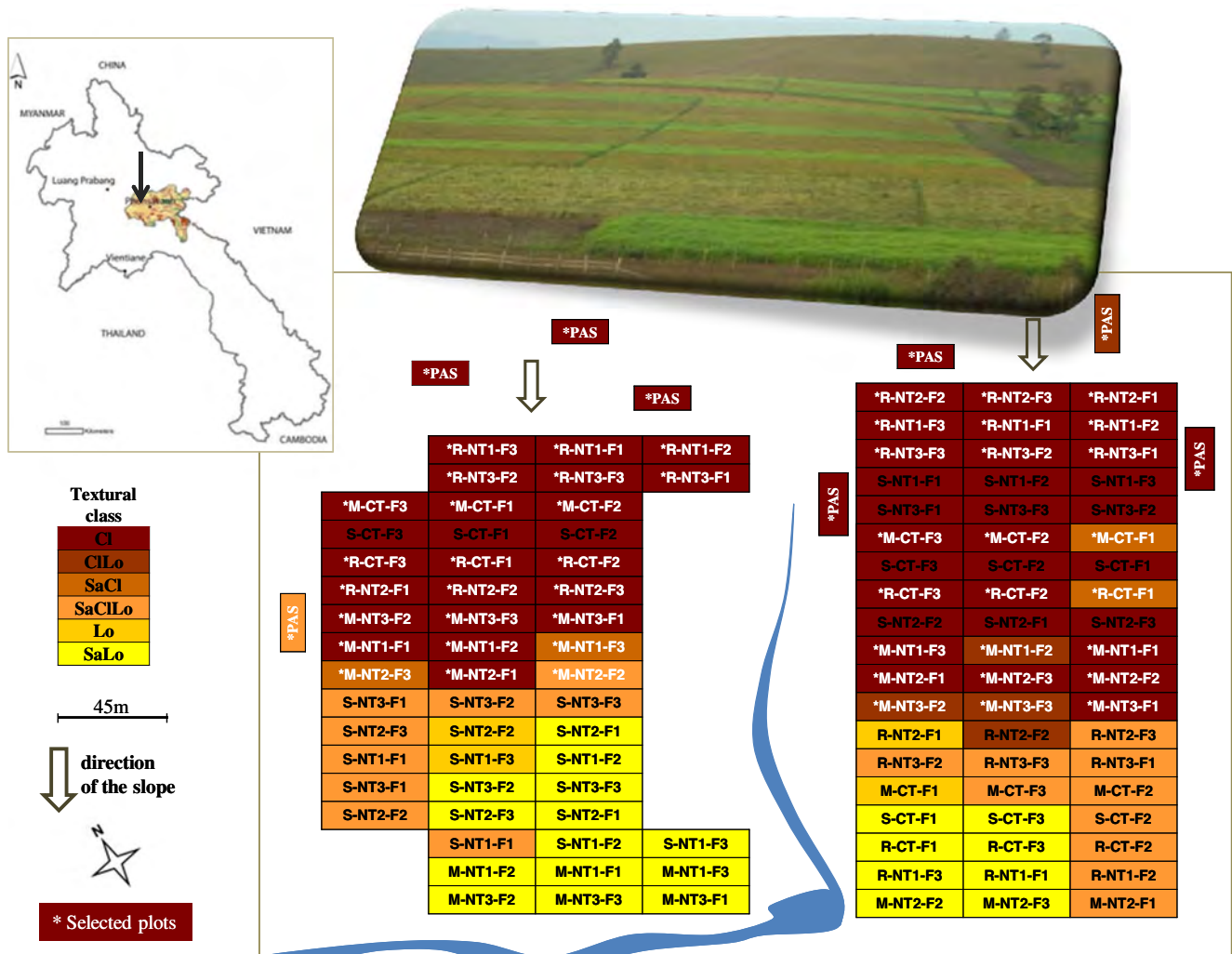


Fig. 1 Experimental design. Starting crops in 2008 (three modalities): *R* rice variety Sebotal; *M* maize hybrid LVN10; *S* soybean variety Asca. Cropping systems (four modalities): *CT* conventional tillage based on annual soil plowing with disks and burying of former crop residues; *NT* (1, 2, and 3), no-till systems with cover crops: finger millet (*Eleusine coracana* Gaern) and pigeon pea (*Cajanus cajan*) associated prior to and with maize in NT1; finger millet and stylo (*Stylosanthes guianensis* cv. CIAT 184) associated prior to and with maize in NT2; ruzi grass (*Brachiaria ruziziensis* cv. ruzi) and pigeon pea associated prior to and with maize in NT3; stylo associated with rice in all NT treatments; oat (*Avena sativa* L.)

and buckwheat (*Fagopyrum esculentum* Moench) in succession of soybean in all NT treatments. Fertilization (three modalities): *F1* 60–80–60 kg ha⁻¹ year⁻¹ of N–P₂O₅–K₂O (N limited to 32 kg ha⁻¹ year⁻¹ for soybean); *F2* 120–160–120 kg ha⁻¹ year⁻¹ of N–P₂O₅–K₂O (N limited to 32 kg ha⁻¹ for soybean); *F3* F2 during the first 2 years, F1 after that; N coming from urea (46 % N), P₂O₅ from thermo phosphate (16 % P₂O₅, 28 % CaO, and 18 % MgO), and K₂O from KCl (60 % K₂O). In addition, all agricultural treatments received an initial application of 2 Mg ha⁻¹ of locally produced lime (27 % of CaO). *PAS* surrounding natural pasture taken as reference treatment. Asterisks treatments selected for the study

highlight the differences related to agricultural management. The natural surrounding pastureland (*PAS*) was taken as reference treatment (eight replicates).

2.2 Estimations of stubble restitutions

We used the cumulated amount of stubble returns as a quantitative indicator of organic inputs, and the percentage of broad-leaf species in crop residues returns as a qualitative indicator of organic inputs (Table 1). Stubble production, including associated crop and weed contributions, were

estimated twice a year: at main crop harvests and before land preparation. Measures were made in each plot on six subplots of 4 m² each randomly chosen. A random lump crop residue sample of 2 kg was taken from the six subplots to determine dry biomass. Grain yields were measured on each total plot area (270 m²).

2.3 Soil sampling

The soil was sampled at 0–10-cm depth, in June 2009, during the second year of the rotational sequence. For soil

Table 1 Top soil (0–10 cm layer) physicochemical characteristics and crop residue returns according to land use management (56 plots)

Tillage system	Clay (%)	Sand (%)	MWD (mm)	Bulk density (g cm ⁻³)	pH H ₂ O (1:5)	SOC (g kg ⁻¹)	Total N (g kg ⁻¹)	C:N	P Olsen (mg kg ⁻¹)	Exch. Al (c mol kg ⁻¹)	Σ base (c mol kg ⁻¹)	Effec. CEC (c mol kg ⁻¹)	Stubble (Mg ha ⁻¹)	Broad leaf (%)
PAS	46±11a	29±13a	8.5±0.6b	1.27±0.03a	5.3±0.2ab	33.8±3.0ab	2.4±0.2b	13.8±0.5a	2.7±0.5a	1.2±0.3c	1.0±0.3a	2.7±0.6 a	5.1±1.0a	<1a
CT	52±6a	33±7a	6.8±1.0a	1.27±0.07a	5.2±0.1a	31.4±1.6a	2.1±0.2a	15.0±0.8b	7.8±2.7b	0.2±0.2b	2.9±0.6b	3.4±0.5 ab	5.6±1.3a	3±2a
NT1	47±6a	35±9a	10.0±0.6c	1.34±0.06ab	5.4±0.2ab	36.5±2.5b	2.5±0.2b	14.7±0.6ab	8.3±2.2b	0.1±0.1a	4.7±1.2c	5.2±1.3 c	10.6±3.1b	18±4b
NT2	46±7a	36±8a	9.7±1.0c	1.36±0.07b	5.4±0.2ab	35.1±4.7b	2.4±0.4ab	14.8±0.9b	8.8±2.2b	0.1±0.1a	4.3±1.2c	4.6±1.2 bc	10.9±3.1b	20±12b
NT3	50±6a	32±7a	10.1±0.9c	1.36±0.04b	5.5±0.2b	36.4±2.6b	2.4±0.2b	14.9±0.5b	9.2±2.4b	0.1±0.1a	4.9±1.3c	5.3±1.5 c	12.8±2.8b	5±2a

Broad-leaf species include weeds, shrubby (e.g., pigeon pea), and thin leaf (e.g., stylo) legumes. Lowercase letters indicate significant differences according to Kruskal–Wallis test ($P < 0.05$), Bonferroni correction

PAS natural pastureland, CT conventional tillage, NT (1, 2, and 3) no-till systems, MWD mean weight diameter of aggregates, SOC soil organic carbon, C/N carbon to nitrogen ratio, Σ base sum of exchangeable bases (Ca, Mg, K, and Na), CEC cation exchange capacity, Stubble 2007 and 2008 cumulated stubble dry matter returns

chemical and microbial analysis, a composite sample was made of a pool of five subsamples taken in the diagonal of the plot (50 m). For soil bulk density and soil aggregate stability determination, randomized triplicates were taken for each plot (total of 36 independent replicates per cropping system and 24 replicates for the natural pastureland).

2.4 Soil physical and chemical analysis

We used the mean weight diameter (MWD) of aggregates as an indicator of soil aggregate stability. Aggregate size classes were separated by wet sieving, following a procedure described by Castro Filho et al. (2002). Briefly, soil samples were passed through a 19-mm mesh sieve, and clods >19 mm were softly broken along their natural cleavage planes and were stored in polystyrene boxes to prevent moisture loss and excessive drying. Samples were then wet-sieved in laboratory through a series of six sieves (8, 4, 2, 1, 0.5, and 0.25 mm). Aggregate separation was achieved by agitation (3.5 cm amplitude, 30 rotations per minute for 10 min). MWD was calculated as follows:

$$\text{MWD} = \sum_{i=1}^n x_i w_i \text{ where } W_i \text{ is the relative weight of each}$$

aggregate class in relation to the whole and X_i is the mean diameter of the considered class (millimeters).

The soil bulk density (ρ_b) was used as an indicator of soil porosity and measured on oven-dried (24 h, 105 °C) undisturbed soil samples by using a 94-cm³ density cylinder.

All soil chemical analyses were done by the CIRAD laboratory in Montpellier, France. Soil organic carbon (SOC) and total nitrogen (N) were used as indicators of soil organic status and quantified by dry combustion. Soil pH (1:5 soil/water slurry), available phosphorus (P) (Olsen method), cation exchange capacity (CEC) (cobalt hexamine chloride reagent), sum of exchangeable bases (Ca, Mg, K, and Na) and exchangeable aluminum (Al) were used as indicators of soil acid–base and nutrient status.

2.5 Soil molecular microbial abundance analysis

We used soil crude DNA concentrations as estimates of microbial biomass since a highly positive linear relationship has been shown between soil DNA recovery and C-biomass measurement, this latter being indicative of the size of microbial biomass (Marstorp et al. 2000; Dequiedt et al. 2011). Soil microbial DNA was extracted and estimated on 2 g (dry weight) of soil using a single procedure developed by Ranjard et al. (2003) and recently optimized and standardized by the GenoSol platform (INRA, Dijon, France). DNA concentrations of crude extracts were determined by electrophoresis in a 1 % agarose gel using a calf thymus DNA standard curve.

We estimated fungal and bacterial densities by real-time quantitative polymerase chain reaction (qPCR) of

ribosomal DNA (rDNA). This approach has recently become a valuable, accurate, and culture-independent molecular tool for quantifying soil bacterial and fungal abundance (Smith and Osborn 2009; Chemidlin Prevost-Boure et al. 2011). We amplified 18S rDNA sequences, which are specific to fungi, on 2.5 ng purified soil DNA using FR1 and FF390 primers and qPCR mix SYBr® Green as described by Chemidlin Prevost-Boure et al. (2011). For bacteria, we amplified a 16S rDNA sequence using primers 341F and 515R and 2 ng purified soil DNA, as suggested by Smith and Osborn (2009). Real-time qPCRs were performed using the Gene Amp PCR System 9700 (Applied Biosystems®).

2.6 Soil bacterial community genetic structure analysis

The bacterial community structure was assessed using the Bacterial Automated Ribosomal Intergenic Spacer Analysis (B-ARISA) method (Ranjard et al. 2003). Briefly, 12.5 ng of DNA was used as a template to amplify the bacterial ribosomal intergenic spacer by PCR. PCR products were purified using the MinElute Kit (Qiagen®) and quantified using Smart Ladder (Eurogentec®). PCR fragments were resolved on a LiCor® DNA sequencer (ScienceTec) under denaturing conditions as described in Lejon et al. (2007). Profiles were analyzed using the 1D-Scan® software (ScienceTec), converting fluorescence data into electrophoregrams, where peaks represented PCR fragments and the height of the peaks the relative proportion of the fragments in the total products. Lengths (in base pairs) were calculated by using a size standard with bands ranging from 200 to 1 659 bp.

2.7 Statistics

The effect of land use management on quantitative parameters was tested by the nonparametric Kruskal–Wallis test performed under XLSTAT software (Addinsoft®). Differences between means were tested by paired multiple comparison with Bonferroni correction ($P < 0.05$). Microbial genetic structure data obtained from the 1D-Scan software were converted into a table summarizing the band presence (i.e., peak) and intensity (i.e., height or area of peak) using the PrepRISA software (Ranjard et al. 2003) under R free software version 2.10.1. Principal component analysis (PCA), between-group analysis, and coinertia analysis were performed using the ADE-4 software (Thioulouse et al. 1997) under R software and provided an ordination of data in factorial maps based on the scores of the first two principal components. Monte Carlo tests were performed with 1,000 permutations to confirm the significance of the discriminated clusters.

3 Results and discussion

3.1 Early effect on top soil physicochemical characteristics

After 2 years of native grassland conversion to agriculture, we observed significant modifications of top soil physical and chemical characteristics. Soil aggregate stability decreased (MWD in Table 1) along the gradient NTs > PAS > CT and was positively correlated with the total amount of stubble restituted, SOC, total soil nitrogen, and fungal and bacterial densities (Fig. 2). Our results highlight the role of fresh plant and root-derived residues, SOC, and microbial-binding agents (e.g., fungal hyphae and polysaccharides) in enmeshing soil particles, in concordance with other studies (Six et al. 2002). Contrary to Bossuyt et al. (2001), we found that the diversity of residues also influenced macroaggregation positively with significant correlations between the mean weight diameter of aggregates and the percentage of broad-leaf species in stubble restitutions (Fig. 2). In addition, the macroaggregate disruption process observed under CT, with aggregate size values decreased by 19 % compared to PAS (Table 1), might be mostly related to the direct action of tilling tools, the aggregates being submitted to compressive and shearing forces during tillage (Balesdent et al. 2000; Six et al. 2002).

Early modifications of top soil porosity were also observed, with higher bulk density values under NT compared to PAS (Table 1) which might be related to the compacting effect of agricultural equipment occurring during crop rolling and sowing operations. This compacting effect was not observed under CT due to the annual tillage which induced soil mechanical fragmentation.

Regarding top soil organic status, a significant decrease of SOC content along the gradient NTs \geq PAS > CT (Table 1) was classically recorded. SOC was positively and highly correlated with soil total nitrogen (N) content, the total amount of stubble returns, the percentage of broad-leaf species in restitutions, and the aggregates' stability (Fig. 2). As for organic status, early modifications of soil acid–base and mineral nutrient status were also observed, with a positive impact of inorganic fertilizer use on soil nutrient availability in cropping system. Indeed, all cultivated treatments showed significant increases in CEC, total exchangeable bases, and available phosphorus (P), as well as a related decrease in exchangeable Al content (Table 1). The comparative analysis of the cropping systems, however, appeared in favor of NTs, with 1.5-fold higher exchangeable bases and CEC content under NTs than under CT (Table 1).

Altogether, the evaluation of the impact of agricultural systems on top soil physicochemical parameters highlighted early but classical modifications in favor of no-till systems which induced a significant increase of top soil aggregate stability and SOC content, and higher nutrients availability.

pH																	
SOC	0.18																
TN	0.05	0.88															
C:N	0.13	-0.07	-0.48														
P	0.33	0.15	-0.09	0.48													
Base	0.48	0.57	0.45	0.05	0.59												
Al	-0.63	-0.30	-0.12	-0.29	-0.61	-0.82											
CEC	0.47	0.59	0.50	-0.01	0.52	0.98	-0.76										
ρ_b	0.26	0.23	0.05	0.21	0.26	0.43	-0.49	0.41									
MWD	0.40	0.45	0.38	-0.02	0.28	0.56	-0.52	0.55	0.28								
Stub	0.46	0.63	0.50	0.02	0.44	0.80	-0.75	0.77	0.43	0.68							
Leg	0.22	0.42	0.20	0.29	0.38	0.59	-0.52	0.52	0.37	0.46	0.48						
qDNA	0.12	-0.01	-0.10	0.13	-0.03	-0.04	0.04	-0.01	0.06	0.15	-0.11	0.12					
B16S	0.21	0.40	0.25	0.07	0.14	0.32	-0.33	0.27	0.26	0.40	0.39	0.49	0.57				
F18S	0.22	0.22	0.02	0.21	0.02	0.13	-0.25	0.09	0.24	0.35	0.24	0.44	0.51	0.83			
F:B	0.07	-0.19	-0.34	0.32	-0.17	-0.30	0.11	-0.29	-0.01	-0.04	-0.21	-0.04	0.02	-0.12	0.36		
	pH	SOC	TN	C:N	P	Base	Al	CEC	ρ_b	MWD	Stub	Leg	qDNA	B16S	F18S	F:B	

Fig. 2 Correlation matrix (Spearman) of the PCA performed on soil physicochemical parameters. Land use management impacts on organic input and microbial abundance variables were added as additive variables (in gray). Values in bold are significantly different from 0 at $P < 0.05$ (52 plots). SOC soil organic carbon, TN total nitrogen, C:N carbon to nitrogen ratio, P available phosphorus, Base sum of

exchangeable bases, Al exchangeable aluminum, CEC cation exchange capacity, ρ_b bulk density, MWD mean weight diameter, Stub total amount of stubble returns, Leg percentage of broad-leaf species in Stub, qDNA molecular microbial biomass, B16S molecular bacterial density, F18S molecular fungal density, F:B fungal to bacterial ratio

However, these early changes did not impact soil productivity with similar crop growth and grain yields observed in 2008 (data not shown), this latter being certainly related to good physical (aggregate stability) and organic (SOC content) characteristics of soils before land reclamation as estimated by the natural pasture characteristics and by the limited time of cultivation at evaluation (2 years).

3.2 Early effect on soil microbial abundance

DNA yields, which were used as estimates of microbial biomass, decreased similarly along the gradient NT1 (mean

value of $16.6 \mu\text{g}$ of DNA g^{-1} of soil) > NT2, NT3, and PAS > CT ($11.9 \mu\text{g}$; Fig. 3a). No significant correlation could be established between soil physicochemical parameters and molecular biomass (Fig. 2). This could be related to the high variability observed within replicates regarding physicochemical and textural characteristics (see standard deviations in Table 1), and microbial biomass (Fig. 3a), which makes the significant discrimination between treatments difficult. This could also be linked to a low sensitivity of the method when confronted with the limited amplitude of soil physicochemical variations in relation with the limited time of cultivation (2 years). Indeed, in more contrasted

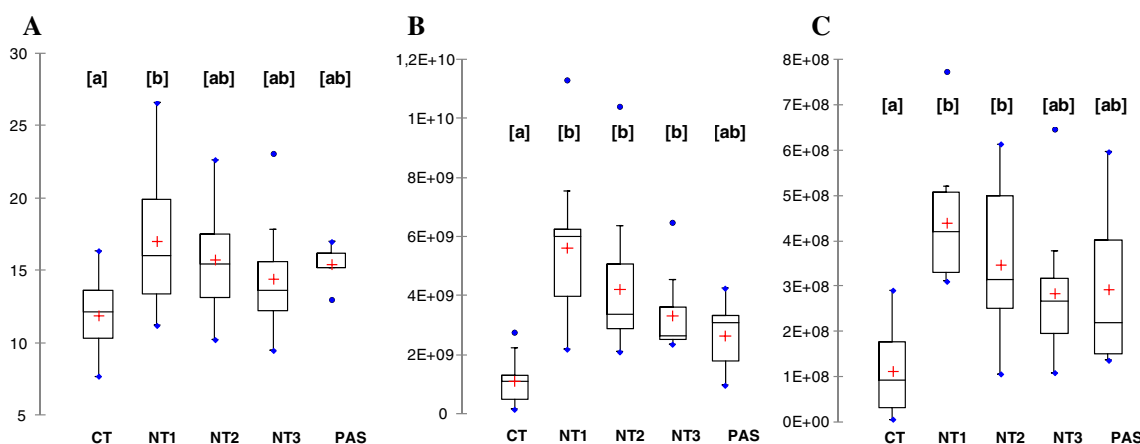


Fig. 3 Top soil (0–10 cm layer) box and whisker representation of **a** molecular biomass (in micrograms of DNA per gram of soil), **b** bacterial density (copy of 16S rDNA per gram of soil), and **c** fungal density (copy of 18S rDNA per gram of soil; 52 plots) recorded in PAS, natural pastureland; CT conventional tillage, and NT (1, 2, and 3) no-till systems. The first (Q1), median, and third (Q3) quartiles are

indicated by the bottom, the central, and the top line of the box, respectively. The bottom whisker extends to the lowest value of the data set, while the top whisker extends to the highest one. Outliers are indicated by points. Letters in brackets indicate significant differences according to Kruskal–Wallis test ($P < 0.05$), Bonferroni correction

situations, SOC and nitrogen content, CEC, and pH have been reported as important parameters influencing microbial biomass (Martiny et al. 2006; Lejon et al. 2007; Dequiedt et al. 2011). Finally, microbial biomass might be more discriminated by other factors than those monitored. Tillage affects soil temperature and humidity which in turn strongly influence soil microbial biomass (Frey et al. 1999; Spedding et al. 2004), and could explain the lowest microbial biomass observed in tilled system.

The number of copies of 16S rDNA and 18S rDNA, which were used as estimates of bacterial and fungal molecular densities, respectively, decreased significantly along the gradient NT1 and NT2 > NT3 and PAS > CT (Fig. 3b, c). After 2 years of cultivation, bacterial and fungal densities were respectively five- and fourfold higher under NT1 than under CT, confirming the high sensitivity and reactivity of these communities to land use management and soil environmental changes (Frey et al. 1999; Lauber et al. 2008; Rousk et al. 2010).

Tillage appears to be a major factor influencing both bacterial and fungal densities with main significant differences observed between tilled (CT) and no-tilled (NTs and PAS) treatments (Fig. 3b, c). Tillage induced a decrease in SOC (Table 1), this latter being positively correlated with bacterial density (Fig. 2). Similarly, tillage induced a reduction of soil aggregate stability and soil macroaggregate content (Table 1), which was also positively correlated with both fungal and bacterial densities (Fig. 2), confirming the importance of macroaggregates as microhabitat for microorganisms (Ranjard and Richaume 2001). In addition, tillage has been demonstrated to affect soil temperature and humidity (Frey et al. 1999), which in turn strongly influence soil microbial abundance and in particular fungal development (Frey et al. 1999; Spedding et al. 2004). Finally, tillage causes direct tissue damage to the fungi leading to a reduction in their abundance at soil surface (Balesdent et al. 2000; Six et al. 2002).

Crop residue diversity appeared as the second main factor influencing both bacterial and fungal densities since the numbers of copies of 16S rDNA and 18S rDNA were significantly and positively correlated with the percentage of broad-leaf species in stubble returns (Fig. 2). The gradient observed regarding the diversity of crop residues, with about 20 % broad-leaf species in restitutions for NT1 and NT2 versus less than 5 % for NT3 and PAS (Table 1), fits with the differences in bacterial and fungal densities observed among no-tilled treatments (Fig. 3b, c). Finally, bacterial density also appeared to be influenced by other trophic parameters with significant and positive correlations found in the total amount of stubble returns and soil exchangeable base content (Fig. 2).

In comparison to the natural pastureland (PAS), the higher amount of stubble returns and soil exchangeable base

content (Table 1) under no-till systems (NTs) might help explain the difference in bacterial density observed between NTs and PAS (Fig. 3b). Altogether, our results highlighted the different processes driving soil bacterial and fungal density changes: bacterial density mainly appeared to be influenced by trophic factors (e.g., diversity and quantity of crop residues, SOC, and sum of exchangeable bases), while fungal density appeared to be influenced by both trophic (e.g., diversity of crop residues) and atrophic factors (e.g., direct tissue damage by tilling tools and soil moisture content).

We did not find any significant difference in the fungal to bacterial (F/B) ratio among treatments (data not shown). F/B ratio is a widely used metric tool to assess the impact of environmental change on soil microbial community structure and functioning (Strickland and Rousk 2009). Our results are not in agreement with Kladivko (2001) who proposed that no-till systems (NTs) would result in a fungal-dominated system instead of the bacterial-dominated system expected under conventional tillage practices. Several studies reported an increase in F/B ratio due to a higher response of fungal biomass to increased soil moisture under NTs (e.g., Frey et al. 1999; Spedding et al. 2004) and/or to the suppression of hyphal growth disturbance by tillage (Frey et al. 1999; Balesdent et al. 2000). On the other hand, our results are in agreement with the review of Strickland and Rousk (2009) who reported empirical evidence in support of such impacts on F/B dominance are still far from generic.

3.3 Early effect on soil bacterial genetic structure

Using principal component analysis with between-group analysis performed on B-ARISA fingerprints, we found four discriminated genetic structure of indigenous bacterial communities under: (1) NT3, (2) NT1 and NT2, (3) CT, and (4) PAS (Fig. 4a), with these four classes being significantly different according to the Monte Carlo test (1,000 permutations, p value < 0.001). A higher discrimination in bacterial communities was observed between non-cultivated and cultivated plots, discriminated on the first axis, than between agricultural treatments, discriminated on the second axis. This distinction between non-cultivated and cultivated soil might increase in the coming years since Buckley and Schmidt (2001) reported higher microbial community structural differences between cultivated and never-cultivated soils, than between cultivated soils showing different cultivation and plant community histories. The low but significant differences in bacterial genetic structure observed between NTs and CT might be related to the limited duration of cultivation (2 years) at evaluation.

Using coinertia analysis between B-ARISA fingerprints and physicochemical parameters, we found that the main

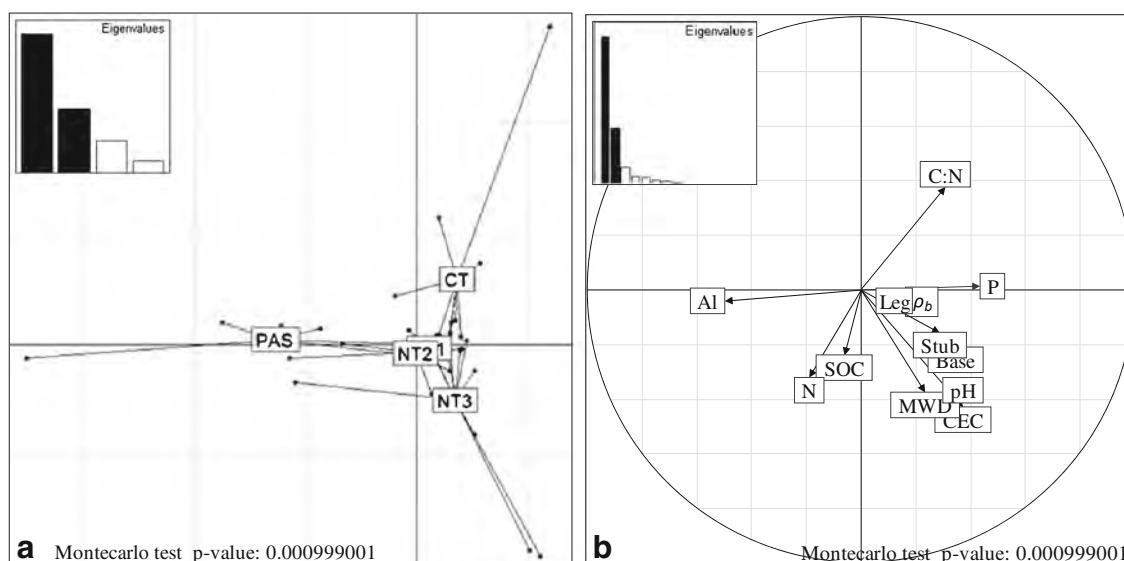


Fig. 4 Factorial maps of: **a** bacterial community genetic structure (principal component analysis of B-ARISA fingerprints, between-group analysis). **b** Correlation circle of the coinertia analysis performed between the PCA of B-RISA fingerprints and the PCA of soil physicochemical characteristics (52 plots). *PAS* natural pastureland, *CT* conventional tillage, *NT* (1, 2, and 3) no-till systems. *SOC* soil organic

carbon, *N* total nitrogen, *C:N* carbon to nitrogen ratio, *P* available phosphorus, *Base* sum of exchangeable bases, *Al* exchangeable aluminum, *CEC* cation exchange capacity, ρ_b bulk density, *MWD* mean weight diameter, *Stub* cumulated amount of stubble returns, *Leg* percentage of broad-leaf species in *Stub*

factors influencing the bacterial genetic structural changes among land use managements (Fig. 4b) were different from those influencing microbial abundance. The first axis of the coinertia factorial map (PC1) showed a significant difference (Monte Carlo test p value <0.001) between non-cultivated and cultivated plots according to soil exchangeable Al and available phosphorus (P), with the structure of the bacterial communities under PAS appearing strongly related to high Al content. The second axis of the factorial map showed an ordination of cultivated soils according to soil pH, nutrient content (CEC, total exchangeable bases), the mean weight diameter (MWD) of aggregates, and soil carbon to nitrogen (C/N) ratio. The early changes in bacterial genetic structure under NT3 were related to an increase in pH, nutrient availability, and MWD as compared to NT1, NT2, and CT (Table 1). By contrast, the early changes observed under CT were more related to a decrease in pH, nutrient availability, MWD and an increase in C/N ratio (Table 1).

Our results are in agreement with several studies that describe pH as the main discriminating factor of bacterial diversity (Grayston et al. 2004; Martiny et al. 2006; Fierer et al. 2009). Variations of C/N ratios have been shown to explain shifts in F/B ratio (Fierer et al. 2009) and bacterial genetic structure in vineyard soils (Lejon et al. 2007). However, no reference was found regarding the effect of soil available P, Al content, and/or CEC changes on bacterial genetic structure. While we show that the quality of crop residues strongly influenced bacterial abundance, this factor

did not appear influential regarding bacterial community structural changes, contrary to other studies (Nicolardot et al. 2007; Pascault et al. 2010). Finally, tillage could partly explain the differences in bacterial genetic structure observed between conservation (NTs) and conventional (CT) cropping systems, by modifying microbial access to crop residues and soil moisture content (Nicolardot et al. 2007).

4 Conclusion

In an acid tropical grassland environment, our results show that no-till (NTs) and conventional tillage (CT) farming systems both induced early but different modifications in top soil properties in reference to the natural pasture (PAS): NTs increased aggregate stability and soil organic carbon content, enhanced nutrient availability and microbial biomass as a result of a simultaneous increase of fungal and bacterial densities. We also showed a significant discrimination of soil microbial community structures between NTs, CT, and PAS. In addition, bacterial abundance and diversity appeared to be differently driven by soil environment changes: bacterial density was affected by the quantity and diversity of crop residues, soil organic carbon, and exchangeable base content, whereas soil bacterial genetic structure was influenced by soil exchangeable Al content, pH, CEC, and soil C/N ratio, all these parameters being affected by tillage, residue management, and soil organic and inorganic amendments. As an application of our results,

we recommend no-till systems with high residue restitutions and lime amendment in order to improve the physical, chemical, and microbial properties of tropical acid soils, and thus contribute to the sustainability of agricultural systems.

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Validation and Application of a PCR Primer Set to Quantify Fungal Communities in the Soil Environment by Real-Time Quantitative PCR

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Abstract

Fungi constitute an important group in soil biological diversity and functioning. However, characterization and knowledge of fungal communities is hampered because few primer sets are available to quantify fungal abundance by real-time quantitative PCR (real-time Q-PCR). The aim in this study was to quantify fungal abundance in soils by incorporating, into a real-time Q-PCR using the SYBRGreen[®] method, a primer set already used to study the genetic structure of soil fungal communities. To satisfy the real-time Q-PCR requirements to enhance the accuracy and reproducibility of the detection technique, this study focused on the 18S rRNA gene conserved regions. These regions are little affected by length polymorphism and may provide sufficiently small targets, a crucial criterion for enhancing accuracy and reproducibility of the detection technique. An *in silico* analysis of 33 primer sets targeting the 18S rRNA gene was performed to select the primer set with the best potential for real-time Q-PCR: short amplicon length; good fungal specificity and coverage. The best consensus between specificity, coverage and amplicon length among the 33 sets tested was the primer set FR1 / FF390. This *in silico* analysis of the specificity of FR1 / FF390 also provided additional information to the previously published analysis on this primer set. The specificity of the primer set FR1 / FF390 for *Fungi* was validated *in vitro* by cloning - sequencing the amplicons obtained from a real time Q-PCR assay performed on five independent soil samples. This assay was also used to evaluate the sensitivity and reproducibility of the method. Finally, fungal abundance in samples from 24 soils with contrasting physico-chemical and environmental characteristics was examined and ranked to determine the importance of soil texture, organic carbon content, C:N ratio and land use in determining fungal abundance in soils.

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Introduction

Soil plays a crucial role in determining the rates and the diversity of ecosystem processes. Indeed, soil houses very large quantities of microorganisms with enormous biodiversity [1–4], resulting in numerous biological interactions and ecological processes. To date, most studies have focused on soil bacteria and analyzed their diversity [5], ecology [6–8], or role in biogeochemical cycles [9,10]. Despite the important role of fungi in ecosystem functioning (*e.g.* nutrient and C cycling) and their huge biodiversity (1.5 million species; [11]), studies of soil fungal communities represent only about 30% of the total investigations of soil microbial communities reported in the literature. In the context of molecular ecology, this trend may be observed because fewer molecular tools are available for the *in situ* characterization of soil fungi [12], the genetic sequence

databases for soil fungi are smaller than those for soil bacteria, and also because fewer groups are working on soil fungi. However, the need to develop new tools to improve our ability to characterize the diversity and abundance of soil fungal communities has been highlighted by the rapid evolution from descriptive to quantitative approaches in microbial ecology. An absolute quantification of soil fungal communities could i) provide a simple bio-indicator for evaluating the impact of human activities on soil; ii) reveal the relative importance of soil fungi, as compared to bacteria, in the total microbial biomass. This result could also be combined to the quantification of specific fungal phyla to estimate their relative abundance. Finally, this would lead to a better understanding of the role of fungi in soil biological functioning.

Real-time quantitative PCR (real-time Q-PCR) has recently become a valuable molecular tool for quantifying indigenous

organisms in environmental samples directly from environmental DNA extracts. This method is powerful, accurate and culture-independent. Different taxonomic levels can be attained by targeting different regions in the genome [13,14] (e.g. “broad” taxonomic resolution with targets located in rrs genes, and finer taxonomic resolution with targets located in more variable regions like the Internal Transcribed Spacer (ITS)). The real-time Q-PCR method has been used successfully to measure total bacterial abundance [14] and the abundance of bacteria involved in the nitrogen cycle in soils [13]. In addition, the suitability of the method for quantifying soil fungal communities has been demonstrated *in vitro*. Raidl et al. [15] demonstrated a linear relationship between the number of copies of the ITS region detected by real-time Q-PCR and the hyphal length of *Piloderma croceum*, an ectomycorrhizal fungus. Nevertheless, real-time Q-PCR still needs to be improved for the study of soil fungal communities. Indeed, when this approach was used in different studies to target the ITS region in fungi in environmental samples [16–23], the reproducibility and accuracy of the real-time Q-PCR measurements of absolute fungal abundance in soil samples were hampered by the length of the ITS region, together with its high length polymorphism and potential resulting taxonomic bias [24]. The reproducibility and accuracy of the method constitute strong limitations in ecological studies of soil fungal communities [25]. These are largely determined by the length of the amplicon produced [14,25]: short amplicon enhance the accuracy and the reproducibility of the method. To overcome these limitations and enhance the reproducibility of the method for soil fungal communities, some studies focused on the 18S rRNA gene region [26–28]. This region was chosen because it contains conserved regions with only slight length polymorphism and because sequence polymorphism is not a limiting factor in the real-time Q-PCR approach. However, limitations related to the length of the targeted region [28] or to the specificity of the primer set [26,27] were still encountered. This highlighted the need to identify other primer sets suitable for use with real-time Q-PCR.

Our aim was therefore to extend the use of a primer set commonly involved in the characterization of soil fungal community composition in the literature, with quantification of the soil fungal community by real-time Q-PCR. The 18S rRNA gene was chosen as the target gene because, conversely to the ITS region, it contains conserved regions unaffected by length polymorphism. To identify and evaluate the suitable primer set, a three step procedure was chosen. First, 33 primer sets targeting the 18S rRNA gene were compared *in silico* for the length of the amplicon, so as to ensure good accuracy and reproducibility of the detection technique. This allowed the selection of a subset of primer sets that produced shorter amplicons than the primer set nu-SSU-0817/nu-SSU-1196 [26], already used in combination with real-time Q-PCR approach. Among this subset, the primer sets were tested for specificity for *Fungi* against a theoretical optimal primer (fully specific of *Fungi*) to identify the best sets. These best sets were then compared in details to one another for their specificity and coverage for *Fungi*. This allowed the selection of the more relevant primer set corresponding to the best consensus between fungal specificity, fungal coverage and amplicon length: FR1/FF390 (targeted gene: 18S rRNA gene, target region length: *c.a.* 350 bp). This primer set was developed a decade ago by Vainio and Hantula [29] for the analysis of wood-inhabiting fungi by Denaturing Gradient Gel Electrophoresis (DGGE), and is frequently used in combination with DGGE to analyze fungal community composition in soil (e.g. [30–32]). Second, a real time Q-PCR run was performed using the primer set FR1 / FF390 on five independent soil samples with serial

dilutions of template DNA. The resulting amplicons were used to validate the fungal specificity of the primer set FR1 / FF390 *in vitro* through a cloning - sequencing approach and the real-time Q-PCR data from this run were used to evaluate method sensitivity and reproducibility. Third, the method was applied to 24 soil samples originating from different physico-chemical conditions and subjected to various land-use practices (forests, grasslands and croplands) to evaluate the ecological potential of this tool and rank the influence of soil properties and land-use practices on soil fungal abundance.

Results and Discussion

In Silico Selection and Validation of the relevant Primer Set

65 unique primers located in the 18S rRNA gene were extracted from the literature and from the AFTOL primer database. They were analyzed *in silico* as 33 primer sets (Data S1). The relevant primer set was selected as the best consensus between fungal specificity, fungal coverage and the length of the amplicon. The 33 primer sets were first discriminated for the length of the amplicon produced (Figure S1) which ranged from 135 bp (± 9 bp) to 523 bp (± 3 bp). This led to the selection of a subset of 23 primer sets that were tested for their fungal specificity against a theoretical optimal primer set (fully fungal specific) through an ascendant hierarchical classification (Data S1) which produced five significant clusters ($P < 0.05$) of primer sets (Figure S2). The best primer sets were clustered with the theoretical optimal primer and were: nu-SSU-0817/nu-SSU-1196, FF390/FR1, nssu897R/nu-SSU-1196 and nssu1088R/SR2.

We detail below the comparison between these 4 “best” primer sets considering non fungal phyla and the *Fungi* kingdom (Figure 1). Similarities with 0 to 3 mismatches were evaluated in the comparison of the four “best” sets, but only the 0 mismatch analysis, *i.e.* the original primer sets, was examined for specificity and coverage of the *Fungi* kingdom. Analyses involving 1 to 3 mismatches were then examined to test the possibility of improving the primer set sequences and enhancing fungal detection without diminishing the specificity of each primer set for *Fungi*.

The *in silico* analysis indicated that only *Eukaryota* sequences were detected by the four “best” sets (Table S1). Except nssu1088R / SR2, the primer sets had a relatively good coverage of *Fungi* kingdom (64.5% to 69.8%) but also punctual matches with some non-fungal groups. FR1 / FF390 is the set that matched the fewer non fungal groups: *Choanoflagellida* (<0.8%, *Choanoflagellida clade-2*), *Mesomycetozoa* (10.8%, *Ichthyosporea* and *Nuclearia* sequences), and some *Metazoa* (<0.3%, *Cnidaria* and *Porifera* sequences); which was not documented in the literature [30,32–34]. Except for *Nuclearia*, none of the non-fungal groups matched by the primer set are found in soils. The FR1 / FF390 primer set would thus be relevant for a robust and specific detection of the soil fungal community. In comparison, the other primer sets matched these groups at similar or higher levels (e.g. *Choaniflagellida*, *Metazoa*), and additional non-fungal groups (e.g. *Cryptophyta*, *Alveolata*, *Oxymonadida*, *Stramenopiles*) potentially found in soils. This led to the conclusion that FR1 / FF390 primer set was more fungal specific than the other 3 primer sets.

At the fungal level, major phyla (*Ascomycota* and *Basidiomycota*) were very efficiently detected by the primer sets FR1 / FF390 and nssu897R / nu-SSU-1196 (ca. 75% to 80% for both phyla and both primer sets). The other sets presented smaller coverage of each group (nssu-1088R/SR2) or a disequilibrium between the two groups (nu-SSU-0817/nu-SSU-1196). The different

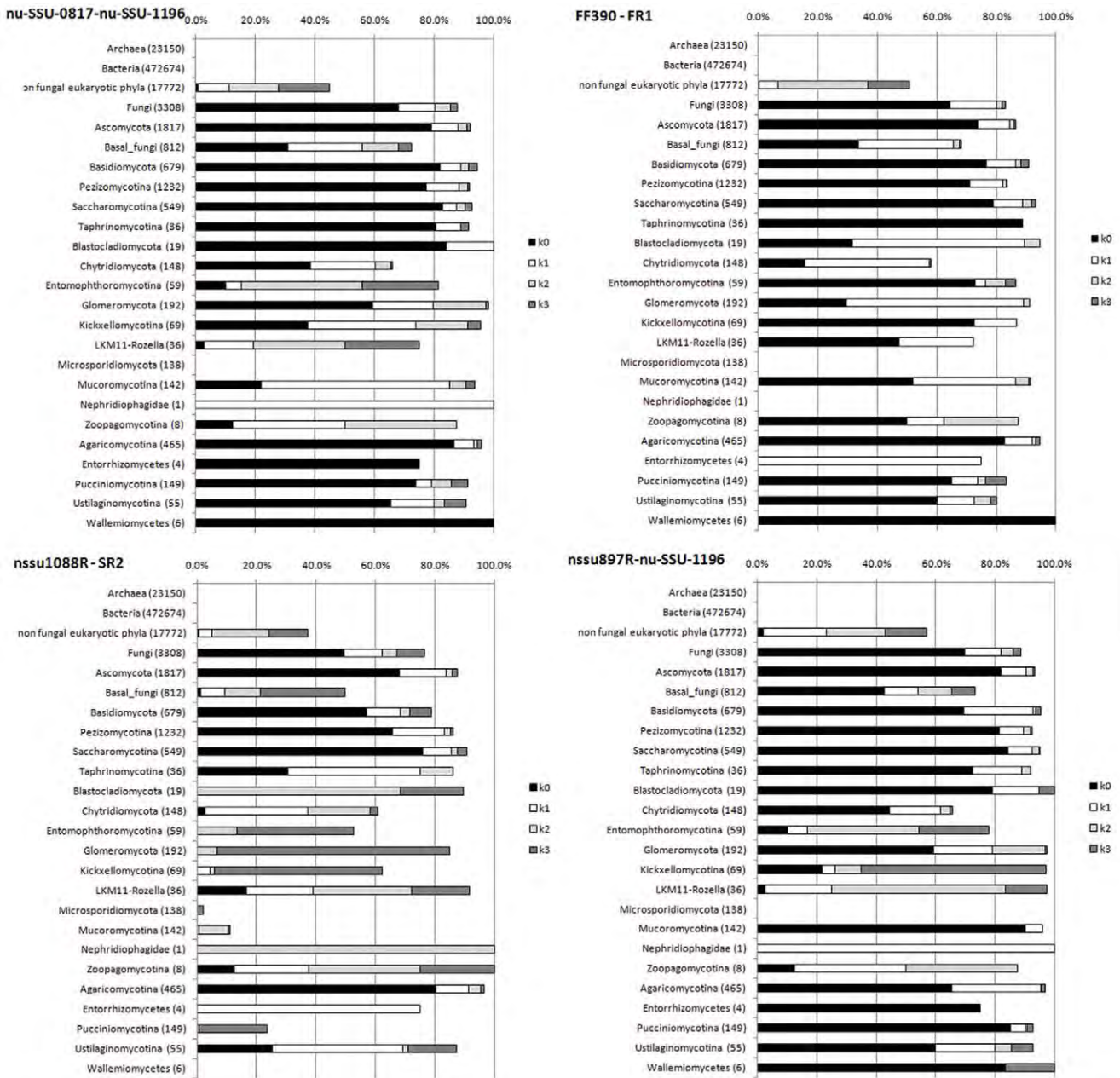


Figure 1. *In silico* comparison of the primer sets nu-SSU-0817/nu-SSU-1196, FR1/FF390, nssu1088R-SR2 and nssu897R-nu-SSU-1196 for their fungal-specificity. For each primer set, k mismatches (0 to 3) were allowed in the *in silico* analysis to test the specificity of the original primer set ($k=0$) and its potential sequence improvement ($k=1$ to 3). For each graph, each bar represents the hit frequency (%) of the primer set for the selected phylum with: $k=0$: black, $k=1$: dark grey, $k=2$: white, $k=3$: light grey. The number of sequences available for a phylum is indicated in brackets. Detailed hit frequencies are provided in Table S1. doi:10.1371/journal.pone.0024166.g001

sub-groups of *Ascomycota* and *Basidiomycota* were also well-covered (coverage ranging from 60% to 87%). None of the four primer sets had a very large coverage of the basal fungal lineages (coverage ranging between 31.0% and 42.6%, except nssu1088R / SR2 with 1.4%). This was mainly determined by the low detection of some basal fungal lineages (*Chytridiomycota*, *Glomeromycota* and *Blastocladales*). The poorer detection of basal fungal lineages, in relation to other fungal groups, is in agreement with the literature [33–35] but shows that the FR1/FF390 and nssu897R/nu-SSU-1196 primer sets are able to take these groups into account, if only

partially. Nevertheless, nssu897R/nu-SSU-1196 remained less fungal specific than FR1 / FF390.

Finally, this analysis showed that the primer set FR1 / FF390 was the best consensus between a short amplicon and a good specificity and coverage of *Fungi*. The original FR1 / FF390 primer set seems better suited for combination with real-time Q-PCR among the sets tested *in silico*. Indeed, it is specific for *Fungi*, matches every major fungal phylum and avoids technical limitations related to target length polymorphism. Even if FR1 / FF390 does not provide fully exhaustive coverage of the fungal

kingdom, the non-covered phyla belong to basal fungal lineages that represent only 1.5–2.0% of the total number of fungal taxa identified to date in the Genbank database [36]. Therefore, soil fungal community abundance should only be slightly underestimated. In addition, the major fungal phyla that might strongly influence estimates of soil fungal abundance are largely and almost equally covered.

For the 4 “best” primer sets, the introduction of mismatches, *i.e.* degenerating primer set sequences into the *in silico* analysis, allowed the test of the potential improvement of their sequences. This effectively increased the hit frequency of the different fungal phyla from 70% to 95% (Figure 1) but decreased the fungal specificity of every primer set with the detection of additional non-fungal organisms to a large extent, particularly *Metazoa*, *Chlorophyta*, *Stramenopiles* or *Cercozoa*. This does not constitute a good compromise for the real-time Q-PCR approach because non fungal sequences cannot be discarded by any post-processing method. Therefore, modifying the sequences in any primer set would have produced biased estimations of soil fungal abundance.

In Vitro Evaluation of the FR1 / FF390 Primer Set

Five soils with contrasting texture, C and N contents, C:N ratio and pH (Table 1), were first used to evaluate the sensitivity, efficiency and reproducibility of the method and thereby define the amount of DNA template to use in the real-time Q-PCR assay on the basis of this information. In a second step, the real-time Q-PCR products obtained directly from these five soils were then cloned and sequenced to validate the specificity of the FR1 / FF390 primer set.

Experimental Determination of the Sensitivity, Efficiency and Reproducibility of the Real-Time Q-PCR Approach

The threshold cycle (C_T) was significantly and linearly related to the logarithm of the starting quantity of 18S rRNA gene copies on the standard curve ($r^2 > 0.99$). This indicates that the method provides accurate estimates of the 18S rRNA gene copy number in pure DNA templates (standard template corresponding to the FR1 / FF390 target region derived from a pure culture of *Fusarium oxysporum* and cloned into PGEMT plasmid). The C_T of the no-templates assay was at least 3.3 cycles higher than that of the most diluted standard (3×10^2 copies of 18S rRNA gene). The sensitivity of the method could therefore be set at 3×10^2 copies of 18S rRNA gene per assay [37]. This detection limit is much lower than that defined for the nu-SSU-0817/nu-SSU-1196 primer set [27] and is within the range of detection limits defined for primer sets targeting the ITS region [17,22].

The efficiency of the real-time Q-PCR method for soil DNA extracts was tested by serial dilution using DNA templates derived from five soil samples. For each soil sample, the relationship between the C_T value and the logarithm of the amount of DNA template in the PCR was linear and highly significant ($r^2 > 0.99$, Figure 2, raw data are provided in Table S6). The PCR efficiencies (derived from the slope of the linear regression) differed from one soil sample to another and ranged between 67% and 103%. They were, however, within the ranges reported in the literature [17,27] and in the same range as the efficiency derived from the standard curve (91%). The observed variations may be related to the different proportions of PCR inhibitors in the samples which vary according to the physico-chemical characteristics of soils. This was supported by the variations in PCR efficiency of each soil sample with DNA template concentration. PCR efficiency was close to the standard PCR efficiency for DNA template quantities of 1 ng to 2.5 ng, except for sample 1101 (73%).

The reproducibility of the method for environmental samples was tested by calculating the coefficient of variation (CV) of C_T and of the number of copies of the 18S rRNA gene throughout the PCR assay for each DNA template quantity. For each soil, the C_T measurements were highly reproducible for a given DNA template quantity within an assay (CV < 2.2%). The lowest ranges of variations in this CV were observed for DNA template quantities ranging from 1 ng to 2.5 ng. Nevertheless, the CV of the number of 18S rRNA gene copies estimated from the standard curve was much higher, ranging from 3% to 23% (Figure 3, except for sample 1051 for which the CV was 49% at the lowest DNA template quantity). This is within the range of CVs reported in the literature [37–39]. The higher CV obtained for the 18S rRNA gene copy number is probably related to error propagation during the conversion of C_T into copy number [37]. The CV values did not seem to be related to template quantity in the real-time Q-PCR mix for a given soil, but the ranges of variation of the 18S rRNA gene copy number were lowest (5% to 16%) for 2.5 ng of template DNA per PCR assay.

According to these results, and because the extractable DNA content of certain soil types may be very low, the DNA quantity in the PCR assay was set at 2.5 ng. This limited the error on the 18S rRNA gene copy number, which ranged from 5% to 16%. It also meant that the PCR efficiency of most templates was close to that of the standards, which ensured the accuracy of the method. The negative controls were below the detection limit set by the standard curve at *ca.* 10^2 copies of 18S rRNA gene per PCR assay.

Table 1. Physico-chemical characteristics of the soil samples used for *in vitro* validation of the FR1/FF390 primer set.

Site number	C _{org} g kg ⁻¹	N	CaCO ₃	P	K %	pH _{water}	Texture	C:N
858	15.0	1.6	BD	0.08	1.5	7.1	Silt Clay	9.4
1012	9.7	1.1	BD	0.10	1.1	7.1	Silt Loam	8.9
1051	26.2	2.5	BD	0.04	3.6	5.4	Sandy Loam	10.4
1101	8.1	0.8	BD	0.09	1.6	6.4	Silt Loam	9.8
1143	60.0	3.0	BD	0.03	3.3	4.3	Sandy Loam	20.3

Texture was determined according to the USDA referential. C_{org}: organic carbon content; N: total nitrogen content; P: available phosphorous; K: total potassium content. BD: below the detection threshold.

doi:10.1371/journal.pone.0024166.t001

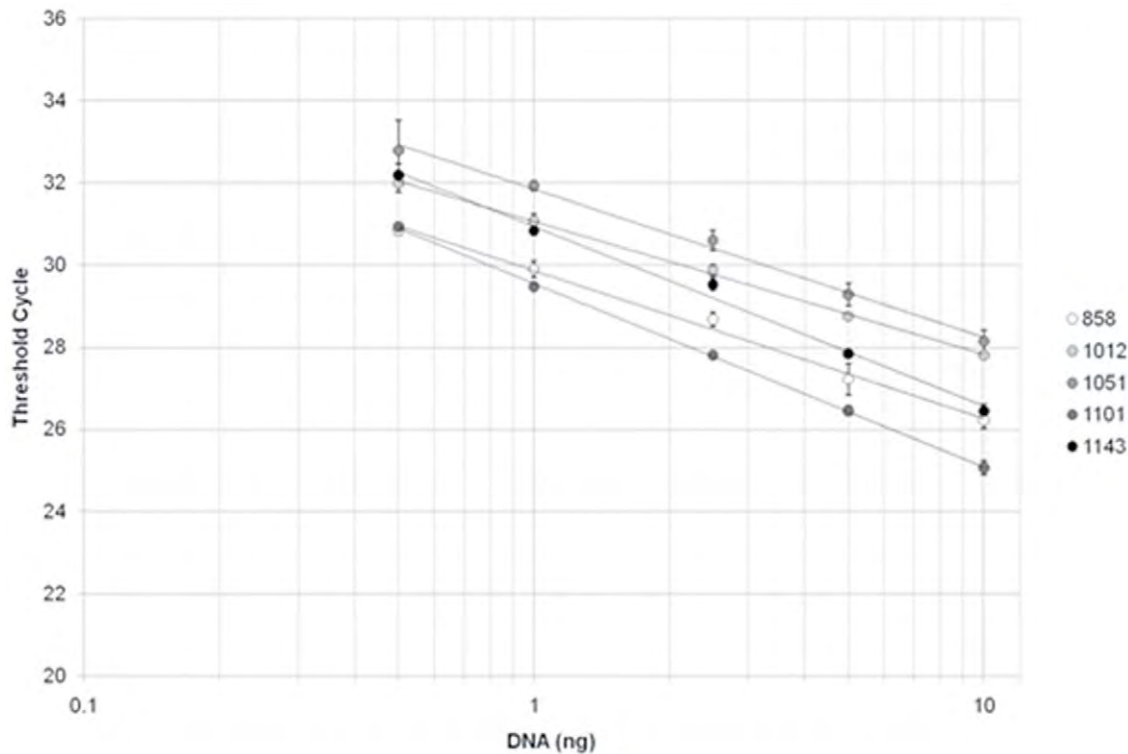


Figure 2. Threshold cycle against DNA quantity in the PCR mix for five soil DNA extracts with serial dilution. DNA quantities are represented in logarithmic scale and correspond to a serial dilution series (10 ng, 5 ng, 2.5 ng, 1 ng, and 0.5 ng). The linear regressions were highly significant ($r^2 > 0.99$) for each soil type. The equations of the regression line were for each soil sample: 858: $y = -3.61x + 29.86$; 1012: $y = -3.24x + 31.06$; 1051: $y = -3.60x + 31.84$; 1101: $y = -4.47x + 29.55$; 1143: $y = -4.37x + 30.94$. doi:10.1371/journal.pone.0024166.g002

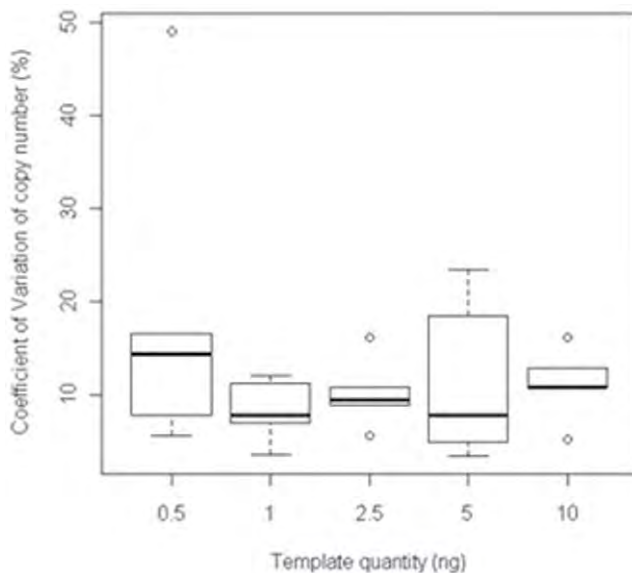


Figure 3. Variation coefficient of 18S rRNA gene copy number with DNA quantity in the PCR mix for five soil DNA extracts with serial dilution. The box limits represent the first and third quartiles of the variation coefficient (CV), the bold line represents the median and the error bars represent the standard deviation. Empty circles correspond to the minimum and maximum of the CV. The CV for each soil was determined from 3 independent measurements. doi:10.1371/journal.pone.0024166.g003

Validation of Primer set Specificity

The real-time Q-PCR products obtained in the above-defined conditions, directly from the DNA of the five soils evaluated for sensitivity, efficiency and reproducibility, were cloned and sequenced to check the specificity of the primer set FR1 / FF390. NCBI-Blast was used for robust affiliation of the sequences. Only fungal sequences were identified (Table S2 for affiliation and accession numbers of the clone sequences). Most of the sequences were successfully affiliated and corresponded to fungal sequences. Six sequences were not completely affiliated but 3 of them were close to *Sordariomycetes* and the 3 others could only be related to eukaryotic fungal sequences. These sequences were aligned with reference sequences extracted from Genbank (Accession numbers in Table S3) to check that the clone sequences clustered according to their affiliation. Figure 4 presents the maximum parsimony dendrogram of the sequences of clones derived from the real time Q-PCR products. The clones did not cluster according to their soil of origin, so any potential bias due to manipulation was limited. The bootstrap values were not significant, due to the length of the sequences (317 to 360 bp), but the different phylogenetic methods tested (Neighbor Joining, Maximum Parsimony and Maximum Likelihood) produced similar clusters, which strengthened the analysis. In addition, the obtained clusters were in agreement with the fungal phylogeny presented in James et al. [36]: basal fungal lineages (groups I, III and IV) were discriminated from *Basidiomycota* (group II_B) and *Ascomycota* (group II_A). Nevertheless, the non-fungal reference sequences did not root the dendrogram and mainly formed a small group with the basal fungal lineages, to which they seem to be closest according to the phylogeny presented in James et al. [36]. The mix of non fungal sequences



- Groups:**
- I
 - IIa: Ascomycota
 - IIb: Basidiomycota
 - III: Zygomycota
 - IV: Basal fungal lineages + non fungal reference sequences
- Soils:**
- ♣: 858
 - ◆: 1012
 - ♥: 1051
 - ♠: 1101
 - : 1143
 - * non fungal reference sequences

Figure 4. Distribution of clones obtained by the cloning-sequencing approach in the different fungal phyla. Symbols indicate the soil of origin of the clone: ♣: 858, ◆: 1012, ♥: 1051, ♠: 1101, ●: 1143. *: Non fungal reference sequences. Genbank accession numbers of clones and their respective affiliation are provided in Table S2. Accession numbers of the non fungal reference sequences are provided in Table S3. doi:10.1371/journal.pone.0024166.g004

with basal fungal lineages was mainly determined by the *a priori* choice of reference sequences. Indeed, construction of the dendrogram of clone sequences alone by maximum parsimony method produced the same clusters and significant bootstrap values (Figure S3).

Clones affiliated to *Ascomycota* (22 clones (HM104488–HM104509) and 3 clones corresponding to fungal environmental samples related to *Sordariomycetes*, group II_A (HM104574–HM104576)), *Basidiomycota* (10 clones (HM104560–HM104569), group II_B) or basal fungal lineages (50 clones of *Zygomycota* (HM104510–HM104559) and 1 clone of *Chytridiomycota* (HM104573) belonging to groups III and IV, respectively), clustered with their corresponding reference sequences. *Microsporidia* rooted the analysis together with 3 clones corresponding to fungal environmental samples (group I, (HM104570–HM104572)) which were not strictly related to any of the above groups. These observations confirmed the affiliation of the cloned sequences and the large spectrum of fungal phyla covered by the primer set, as indicated by our *in silico* analysis. This large spectrum of detection is in agreement with other studies that sequenced the DGGE bands derived by using the FR1 / FF390 primer set on soil DNA extracts [30,33–35]. However, in our case, the clones belonging to basal fungal lineages were much more abundant than would be expected from our *in silico* analysis, the DGGE studies [34,35] or mass sequencing studies [40,41]. This was mainly due to high frequency of similar sequences in our clone library (e.g. 29 identical sequences of *Mucoromycotina*) constituting in only 6 OTUs at the 1% similarity level. Despite similar hit frequencies in the *in silico* analysis, *Ascomycota* sequences were also more abundant than *Basidiomycota* sequences in this study, which were distributed into 20 and 10 OTUs at the 99% similarity level, respectively. Differences in clone number may be related to the soil types selected to test the specificity of the FR1 / FF390 primer set. Indeed, most of the five soils had C:N ratios lower than 10 and relatively high phosphorus (P) contents which could have increased the abundance of *Ascomycota* phyla rather than *Basidiomycota* phyla, according to the results of Lauber et al [19].

No *Nuclearia* sequences were detected in the clones, conversely to what was expected from the *in silico* analysis. This is in agreement with other studies involving this primer set in which only fungal sequences were detected [30,33,35,42] and may be related to a relatively low abundance of *Nuclearia* (only 9 taxa recorded in the Genbank database).

No *Glomeromycota* was detected *in vitro* despite its potential amplification according to the *in silico* analysis. The absence of clones belonging to this particular fungal group may be explained by i) the low relative abundance of this group compared to major groups like *Ascomycota* or *Basidiomycota*; ii) the ecology of this group (mainly symbiotic and rhizospheric fungi according to Pivato et al. [43]) with regard to our soil samples (bulk soil containing mainly sporitic forms) which might limit their accessibility for cell lysis and DNA extraction, and/or iii) competition during amplification. In view of their important role in ecosystem functioning [9], a real time Q-PCR assay was run on DNA extracts from pure cultures of *Glomus versiforme*, *Glomus clarum*, *Glomus claroideum*, *Glomus geosporum* and on *Glomeromycota* rich rhizospheric soil DNA extracts [43]. The significant amplification of *Glomus sp.* (Table S4) demonstrated that the previously observed absence of *Glomeromycota* sequences was not related to the specificity of the FR1 / FF390 primer set, but more probably to their low abundance in bulk soil. To test this hypothesis, we applied our primer set and PCR conditions to rhizosphere soil of *Medicago truncatula* which is rich in *Glomeromycota* and detected a positive signal (Table S5; [44]), confirming our hypothesis.

We were able to conclude from the results of this *in vitro* analysis that the FR1 / FF390 primer set was fungi-specific and gave reliable results by amplifying the various fungal groups irrespective of their proportions in the soil samples. Indeed, the matching of particular fungal groups seems to depend mainly on their ecology, which determines their accessibility for DNA extraction, rather than on primer specificity.

Ecological Validation of Real-Time Q PCR for Fungi

The developed method was applied to 24 independent soils of contrasting physico-chemical characteristics and land-use type (Table 2). The aim was to evaluate the combined use of FR1 / FF390 and real-time Q-PCR for ecological investigation by focusing on the determinism of the quantitative variation of fungal abundance in soil. The abundance of soil fungal communities estimated from the 18S rRNA gene copy number was not converted into “number of fungal cells per gram of soil” because many types of fungi are multinucleate cells with very variable numbers of nuclei per cell between species.

Fungal abundance ranged from $6.9 \cdot 10^6$ to $2.1 \cdot 10^9$ copies of 18S rRNA gene $\cdot g^{-1}$ of soil (Figure 5, raw data are provided in Table S7) and was significantly correlated with soil physico-chemical properties (Table 3). Fine-textured soils exhibited a higher fungal abundance than coarse-textured soils, fungal abundance being negatively correlated with fine sand content. This observation is in agreement with the large number of bacterial and fungal organisms, as well as the greater microbial biomass, generally observed in silt or clay soils [45–47]. Fine-textured soils provide a more favorable habitat for microbial growth than coarse soils, offering better protection from desiccation, gas diffusion, toxic exogenous compounds and predation by protozoa [48]. Furthermore, the availability of carbon and nitrogen nutrient resources for indigenous microbes is generally higher in fine-textured soils due to better protection of the organic matter [49]. In addition, fungal abundance was significantly and positively correlated with C_{org} content and C:N ratio. This confirmed the major contribution not only of the availability of organic matter but also of its biochemical quality, the C:N ratio reflecting the recalcitrance of soil organic matter to microbial degradation, in agreement with other studies on soil fungal biology [9,19,46,50,51]. This would accord with the trophic niche differentiation between bacteria and fungi proposed by de Boer et al [9]. According to these authors, fungi preferentially decompose complex organic matter (cellulose, lignin) and interact with soil bacteria through co-metabolism of the fungal exudates. Under these conditions, an accumulation of complex organic matter would promote fungal development and increase fungal abundance (the case in this study as C_{org} and the C:N ratio are correlated; $r = 0.54$; $P < 0.05$). Nevertheless, this hypothesis needs to be tested by sampling on a larger scale under a broader range of C_{org} and C:N ratio conditions. It has been demonstrated that other soil parameters may also be involved in determining the abundance of the soil fungal community: e.g. pH [52], P content [19] or N content [16,53,54]. These observations were not confirmed in this study. This could be i) because the gradients of pH, P content and N content between the different ecosystems were too small, thus preventing the observation of significant trends in the response of soil fungal abundance to these parameters, or ii) because different fungal phyla may respond differently to these parameters (e.g. Phosphorous, in Lauber et al [19]), or iii) because these parameters interacted with each other to influence the abundance of the soil fungal community.

Figure 5 shows the number of 18S rRNA gene copies for different land-use types, i.e. forests, croplands and grasslands. Forest sites contained a significantly ($P < 0.05$) higher average

Table 2. Physico-chemical characteristics and land-use of the soil samples used for ecological validation of the FR1/FF390 primer set combined with a real-time Q-PCR approach.

Site number	C _{org} ** g kg ⁻¹	N	CaCO ₃	P***	K %	pH _{water}	Texture	C:N***	Land Use
634	19.0	1.8	190.0	0.1	0.5	8.3	Clay loam	10.6	Cropland
750	26.4	2.8	77.6	0.04	1.6	8.0	Clay	9.4	Cropland
854	10.5	0.9	BD	0.08	0.5	6.7	Loam	11.5	Cropland
914	34.4	3.6	56.8	0.1	1.5	7.9	Silt Clay	9.5	Cropland
917	18.8	1.8	BD	0.07	1.6	7.0	Silt Clay Loam	10.3	Cropland
968	11.5	1.2	BD	0.13	1.1	7.0	Silt Clay Loam	9.3	Cropland
1220	9.8	1.0	BD	0.04	3.5	5.9	Loamy Sand	9.9	Cropland
1224	25.6	2.5	6.0	0.03	1.7	7.8	Clay	10.1	Cropland
633	32.8	2.5	39.9	BD	0.4	7.7	Clay	13.0	Forest
693	24.3	1.6	BD	0.02	0.7	4.6	Loam	15.2	Forest
807	26.1	1.5	BD	BD	1.2	5.4	Silt Clay Loam	17.8	Forest
810	42.6	3.0	41.2	0.01	1.2	7.7	Silt Clay	14.3	Forest
857	85.4	6.0	239.0	0.02	0.7	8.0	Silt Clay Loam	14.3	Forest
910	99.5	6.4	47.2	0.02	1.2	7.4	Clay	15.5	Forest
1004	56.8	4.2	130.0	BD	1.4	7.8	Clay	13.6	Forest
1053	42.8	2.8	BD	0.02	2.4	5.0	Loam	15.1	Forest
907	25.0	2.4	BD	0.03	0.9	5.9	Loam	10.3	Grassland
963	23.7	2.3	BD	0.02	2.5	5.6	Loam	10.4	Grassland
965	42.8	4.5	BD	0.02	2.0	6.8	Silt Clay	9.4	Grassland
1095	29.2	2.9	1.3	0.02	1.8	6.9	Clay	10.0	Grassland
1099	21.8	2.1	106	0.04	2.0	8.1	Clay	10.2	Grassland
1146	9.9	0.9	BD	0.04	1.0	6.1	Silt Loam	10.6	Grassland
1182	17.1	1.9	BD	0.04	3.7	5.5	Sandy Loam	8.9	Grassland
1305	18.3	1.8	BD	0.02	3.5	6.5	Loam	10.3	Grassland

Texture was determined according to the USDA referential. C_{org}: organic carbon content; N: total nitrogen content; P: available phosphorous; K: total potassium content. *, **, ***: significant differences between Land Use type for edaphic parameters: P<0.05; P<0.01; P<0.001; respectively (Kruskal-Wallis non parametric test). BD: below the detection threshold.

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number of copies of 18S rRNA gene.g⁻¹ of soil (6.9 10⁸ copies.g⁻¹ of soil) than grassland and cropland sites, which did not differ significantly from each other, (9.5 10⁷ and 1.9 10⁸ copies.g⁻¹ of soil, respectively). This difference was significant despite the greater variability in the number of 18S rRNA gene.g⁻¹ of soil observed in forest sites, as compared with grassland and cropland sites, (coefficients of variation: 87%, 78% and 50% for forest sites, cropland sites and grassland sites, respectively). These results are in agreement with those reported in the literature [17,27]. The greater abundance of fungi in forest sites, compared to croplands and grasslands, may be related to their higher C:N ratio and higher C_{org} content, showing that interactions between soil characteristics and vegetation type may also affect soil fungal abundance [55]. In addition, the high variability of soil fungal abundance in forests may partly be due to variations in the response of fungal groups to the C:N ratio (positive or negative correlation, [19]) and the multiple symbiotic associations between plants and soil fungi. On the other hand, the higher number of copies of 18S rRNA gene g⁻¹ of soil observed in croplands, as compared to grasslands, might be due to the higher P content of croplands. Indeed, significant differences in P content were observed between these land-use types and P content has been shown to influence the abundance of fungal populations in soil

[19]. The identification of numerous edaphic variables influencing soil fungal abundance, in agreement with the literature, demonstrates that our tool is valid and operational for studying the determinism of fungal abundance in soil.

Conclusion

In conclusion, the FR1 / FF390 primer set should facilitate the quantification of fungi in soils. Our results provide technical and ecological validation of combining use of the FR1 / FF390 primer set with a real-time Q-PCR approach and SYBRGreen® technology, to estimate fungal abundance in soils. The FR1 / FF390 primer set is the best consensus between fungi-specificity, coverage and a short amplicon among the different primer sets tested *in silico*, provides estimates of fungal abundance which are at least as accurate and reproducible as other primer sets in the literature, and avoids the reproducibility limitations associated with length polymorphism associated with the ITS region. Nevertheless, as with other primer sets, the true fungal abundance may be slightly underestimated because of incomplete coverage of the *Fungi* kingdom. However, this underestimation should remain weakly significant because it is related mainly to basal fungal lineages which constitute a small proportion of the fungal taxa currently referenced in the fungal databases. The major fungal

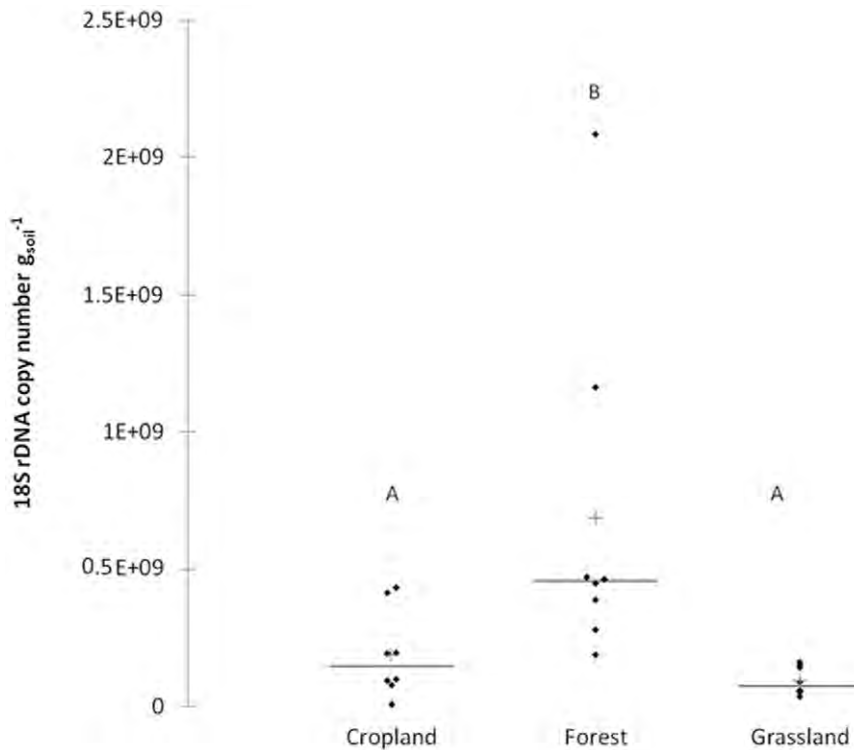


Figure 5. Variations of 18S rRNA gene copy number with land use type for 24 soil samples. Each dot represents the average 18S rRNA gene copy number for one soil sample. Cross and horizontal bars represent the mean and median 18S rRNA gene copy number for the land use type, respectively. Superscript letters indicate significant differences in copy numbers between land use ($P < 0.05$). doi:10.1371/journal.pone.0024166.g005

taxa are equally and almost completely covered. In addition, this primer set is suitable for studying the determinism of soil fungal abundance. The analysis of 24 soil samples showed that the main determinants of soil fungal abundance, in this study, were soil type and land use. All these observations demonstrate that our tool is valid and operational for studying fungal abundance determinism in soil. However, to fully identify such determinism, it now needs to be applied to a large-scale soil sampling scheme, *e.g.* the

European soil survey (particularly in France, UK, Holland and Germany [2]).

Materials and Methods

Soil Sampling

Soil samples were provided by the Soil Genetic Resource Center (platform GenoSol, http://www.dijon.inra.fr/plateforme_genosol, [56]) and were obtained from the soil storage facility of the RMQS (“Réseau de Mesures de la Qualité des Sols” = French Monitoring Network for Soil Quality), which is a soil sampling network based on a 16×16 km systematic grid covering the whole of France [57]. The RMQS consists of 2,195 monitoring sites which have been geo-positioned. Soil profile, site environment, climatic factors, vegetation and land-use were described. Soil samples were air dried under controlled conditions (30°C, hygrometry) and then conserved at -40°C prior to DNA extraction. Five of these soils, contrasting in terms of texture, C and N content, pH and land-use were used to validate primer specificity under real-time Q-PCR conditions (soil characteristics reported in Table 1) and to test the reproducibility of the method. Separately, 24 other independent soil samples were analyzed to test the sensitivity and ecological potential of this tool by ranking the influence of soil properties and land-use practices on soil fungal abundance (physico-chemical characteristics provided in Table 2).

Several physico-chemical parameters were measured on each soil *i.e.*, particle-size distribution, pH water, organic carbon content (C_{org}), N, C:N ratio, soluble P contents, $CaCO_3$, CEC and exchangeable cations (Ca, Mg). Physical and chemical analyses were performed by the Soil Analysis Laboratory of INRA (Arras, France) which is accredited for soil and sludge analysis and

Table 3. Pearson’s correlation coefficients of 18S copy number and physico-chemical parameters.

Variable	18S (copies g^{-1} of dry soil)
Fine Sand ($g\ kg^{-1}$)	-0.41*
C_{org} ($g\ kg^{-1}$)	0.49*
C:N	0.54*
N ($g\ kg^{-1}$)	0.36
Clay ($g\ kg^{-1}$)	0.32
Fine Loam ($g\ kg^{-1}$)	0.29
pH _{water}	0.20
$CaCO_3$ ($g\ kg^{-1}$)	0.10
Coarse Loam ($g\ kg^{-1}$)	0.02
P ($g\ kg^{-1}$)	-0.22
K (%)	-0.22
Coarse Sand ($g\ kg^{-1}$)	-0.30

*: $P < 0.05$.

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recognized by the French Ministry of Agriculture. Land-use was recorded according to the coarse level of the CORINE Land Cover classification (IFEN, <http://www.ifen.fr>) and consisted, for this study, of a rough descriptive classification into three classes: forest, crop systems and grassland.

DNA Extraction and Purification from Soil Samples

For each soil sample, the equivalent of 1.5 g of dry soil was used for DNA extraction, following the procedure described in Ranjard et al. [58] and optimized by platform GenoSol (INRA, France, [56]). Briefly, extraction buffer (100 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl and 2% (w/v) SDS) was added to the sample in the proportion 3:1 (v/w), with two grams of glass beads (106 µm diameter) and eight glass beads (2 mm diameter) in a bead-beater tube. All beads were acid washed and sterilized. The samples were homogenized for 30 s at 1600 rpm in a mini bead-beater cell disruptor (Mikro-dismembrator, S. B. Braun Biotech International), incubated for 30 min at 70°C in a water bath and centrifuged for 5 min at 7000 g and room temperature. The supernatant was collected, incubated on ice with 1/10 volume of 3 M potassium acetate (pH 5.5) and centrifuged for 5 min at 14000 g. DNA was precipitated with one volume of ice-cold isopropanol and centrifuged for 30 min at 13000 rpm. The DNA pellet was washed with ice-cold 70% ethanol and dissolved in 100 µl of ultra pure water. The amount of crude DNA was determined by electrophoretic migration on a 1% agarose gel. The resulting DNA amount was reported to the amount of dry soil to determine the concentration of DNA in ng g⁻¹ of dry soil.

For purification, aliquots (100 µL) of crude DNA extracts were loaded onto PVPP (polyvinyl polypyrrolidone) minicolumns (BIORAD, Marne la Coquette, France) and centrifuged for 4 min at 1000 g and 10°C. This step was repeated if the eluate was opaque. The eluate was then collected and purified for residual impurities using the GeneClean Turbo kit as recommended by the manufacturer (Q Biogene®, France).

Primer Set FR1 / FF390

The primer set FR1 (5'-AICCATTC AATCGGTAIT-3') / FF390 (5'-CGATAACGAACGAGACCT-3') was developed by Vainio and Hantula [29]. This primer set is located at the end of the SSU 18S rRNA gene, near the ITS1 region, and has been shown to be appropriate for DGGE analysis of wood-inhabiting fungal communities. PCR amplification with this primer set produces PCR fragments of ca. 390 bp, suitable for real-time quantitative PCR, with only slight variations due to small length polymorphism.

In Silico Analyses. Dedicated C and Python programs (R. Christen, personal communication) were developed to analyze the different primer sets (primer sets detailed in Data S1, Sheet 01_Primer_list"). We used these programs to search large DNA sequence databases (such as 1 million SSU rRNA sequences) for the presence of primers, including degeneracies as coded by the IUPAC rules and also additional mismatches in order to test the primer improvement. The sequences investigated were Silva [59], direct extraction of every SSU rRNA sequence from EMBL using acnuc [60] and a dedicated reference database of 18S eukaryotic sequences which have been thoroughly analyzed and annotated (<http://keydatatools.com>, [61]).

First, a series of 18S sequences of fungi containing most of the 65 unique primers was retrieved from the Silva database. Primers were aligned in order to precise their locations (column "position"; Data S1, Sheet "02_Primer_selection"). Next, analyses using the databases described above allowed the evaluation of each primer individually for their yield for fungi with 0, 1, 2 and 3 mismatches

and to select a subset of "good" primers. The selection criterion was the ratio between the number of sequences matched at k=2 and k=0. This ratio measures whether the primer detected significantly more fungal sequences with two mismatches than with no mismatch. A well designed primer was therefore a primer that has a small ratio k2/k0 (threshold set at 1.2), because it cannot be improved using more degeneracies. Primers with large ratio k2/k0 were discarded from the following analyses. A good primer is a primer that binds with a high percentage to every fungal clade but to a much lower extend to non fungal clades.

Second, the selected primers were combined into 33 primer sets. The relevant primer set was selected according to the length of the amplicon produced, its specificity and coverage for *Fungi*. A subset was derived from these 33 primer sets according to the length of the amplicon produced that should be short [14] to enhance the accuracy and the reproducibility of the method (Data S1, Sheet "03_Selected_sets"). The threshold was determined by the length of the amplicon produced by the primer set nu-SSU-0817/nu-SSU-1196, a primer set previously used in combination with real-time Q-PCR: 384 bp. This resulted in the selection of a subset of 23 primer sets that were tested for their specificity and coverage for *Fungi* with exact match (Data S1, Sheet "04.1_Sets_evaluation"). This was performed on the Silva Reference sequence database (release 102) to check if the primer sets would not match bacterial or archaeal groups (495,824 Reference Sequences for the SSU genes) because these are well checked, unlike Eukaryotic sequences. Our own well-annotated database of 21,080 eukaryotic SSU rRNA gene sequences was used to check that no fungal group would be missed and also to see if other eukaryotic phyla could be detected by the different primer sets. Note that some sequences which were very short were not used. The yield for each primer set was retrieved and primers were compared to a theoretical optimal primer set (matching only fungal sequences and every fungal sequence) to determine which primer sets would be the more specific and would have the best coverage of *Fungi*. This was done through an ascendant hierarchical classification on the pearson's correlation coefficient similarity matrix based on centred and scaled data (raw data provided in Data S1, Sheet "04.2_Sets_evaluation_HAC"). The best primer sets that clustered with the theoretical optimal primer set were: nu-SSU-0817/nu-SSU-1196; FF390/FR1 ; nssu897R/nu-SSU-1196 and nssu1088R/SR2.

Among these four primer sets, the specificity for *Fungi* was checked in details to determine which one is best for the real-time Q-PCR approach. Different numbers of mismatches (0, 1, 2, 3) were allowed in the analysis to see if the primer set sequences to be used in real-time Q-PCR could be improved : a primer set can be improved if inserting mismatches significantly increases the hit frequency in the targeted phylum without increasing the hit frequency of non-targeted phyla.

Real-Time Q-PCR Conditions. For each soil DNA extract, the real-time Q-PCR products were amplified on an ABI PRISM 7900HT (Applied Biosystems, France) using SYBRGreen® as detection system in a reaction mixture of 20 µl containing 1.25 µM of each primer, 500 ng of T4 gene 32 protein (Appligen, France), 10 µl of SYBR Green PCR master mix, including HotStar Taq™ DNA polymerase, QuantiTec SYBR Green PCR Buffer, dNTP mix with dUTP, SYBR Green I, ROX and 5 mM MgCl2 (QuantiTec, SYBR Green PCR Kit, QIAGEN, France), 2 µl of template DNA, and DNase – RNase-free water to complete the final 20 µl volume.

The real-time Q-PCR conditions consisted of an initial step of 600 s at 95°C for enzyme activation, a second step corresponding to the PCR cycle (40 cycles) with 15 s at 95°C, 30 s at 50°C for hybridization, and an elongation step of 60 s at 70°C. Data were

acquired at the end of this elongation step. A final step was added to obtain a specific denaturation curve from 70°C to 95°C with increments of 0.2°C s⁻¹. Purity of the amplified products was checked by observation of a single melting peak and the presence of a single band of the expected length on 2% agarose gel stained with ethidium bromide. Real-time Q-PCR products obtained from DNA from a pure culture of *Fusarium oxysporum* 47 (INRA Dijon fungal collection) were cloned in a plasmid (pGEM-T Easy Vector System, Promega, France) and used as standard for the real-time Q-PCR assay after quantification with a Biophotometer Plus (Eppendorf, Germany). As purified soil DNA extracts may still contain PCR inhibitors, serial dilutions of the DNA templates (obtained from the 5 soils used to validate FR1 / FF390 primer set specificity) were used to determine the amount of DNA to be used in the real-time Q-PCR assay. The quantities of purified DNA used per well were 10 ng, 5 ng, 2.5 ng, 1 ng, and 0.5 ng.

Clone Library Construction and Sequencing. The PCR products obtained from the five soils used to set up the real-time Q-PCR conditions (template quantity: 2.5 ng) were cloned into the pGEM-T Easy Vector System (Promega, France) according to the manufacturer's instructions. Eighty-nine clones, distributed across the 5 soil samples (number of clones per sample: 7 to 38), were isolated. The DNA of each clone was extracted by "heat/cold" shocks. The plasmid inserts from each clone were amplified using Sp6 and T7 primers. The amplicons were run in 1.5% w/v agarose gel to determine the length of the insert. The inserts were sequenced using the SP6 primer (Cogenics, Meylan, France) and the resulting sequences were deposited in GenBank under the accession numbers referenced in Table S2.

Sequence Identification. Clone sequences were cleaned of plasmid sequence fragments (VecScreen, GENBANK) and affiliated using NCBI-Blast [62].

The distribution of clones sequences in different fungal groups was evaluated by aligning the sequences against reference sequences (Table S3) using seaview [63] and the maximum parsimony tree was computed using Phylowin [63] and visualized with the Dendroscope program [64].

Statistical Analysis

The number of 18S rRNA copies ng⁻¹ of DNA derived the real-time Q-PCR measurements were converted to a number of 18S rRNA copies g⁻¹ of dry soil to allow the comparison between soil samples. A Kruskal-Wallis test was applied to check for significant differences in 18S rRNA gene copy number between the soils. Land-use types were compared with each other by multiple pair comparison. Correlations between soil physico-chemical characteristics and fungal 18S rRNA gene copy number were investigated by applying Pearson's correlation coefficient to the raw data. The significance level was set at the 5% probability level.

Supporting Information

Figure S1 Amplicon length distribution for the 33 primer sets tested in the *in silico* analysis. Red dashed line represents the amplicon length threshold set by the primer set nu-SSU-0817/nu-SSU-1196. A primer set was selected for the next steps if the *in silico* analysis if its amplicon length was below the threshold limit.

(TIF)

Figure S2 Hierarchical ascendant classification of the primer sets. Dotted line: significance threshold at the 5% probability level. Clusters above the threshold limit are significant.

(TIF)

Figure S3 Distribution of clones obtained by the cloning-sequencing approach in the different fungal phyla without introducing reference sequences. Numbers on dendrogram branches are bootstrap values. Colors correspond to the phyla to which clones were affiliated as documented in Table S2.

(TIF)

Table S1 Detailed hit frequencies (%) of the *in silico* analysis of FR1/FF390 and nu-SSU-0817/nu-SSU-1196 primer sets for Bacteria, Archaea, Eukaryota, eukaryotic phyla and fungal phyla. The analysis allowed k mismatches, k ranging from 0 (original primer set sequences) to 3 (test of primer set sequences improvement).

(DOC)

Table S2 Clone sequences affiliation, sequence length and accession numbers in GENBANK database. na: not available.

(DOC)

Table S3 Affiliation and accession numbers of reference sequences from GENBANK database.

(DOC)

Table S4 Glomeromycota amplification on pure culture DNA extracts by real time Q-PCR in combination with FR1/FF390 primer set. NAN: Not A Number. The concentration of DNA extracts from pure cultures of *Glomus sp.* was not determined because very small volumes were available. This precluded having accurate estimates of the number of 18S rRNA gene copies in *Glomus sp.* extracts in this test. Nevertheless, the aim of this test was only to check if *Glomus sp.* DNA was amplified by the primer set FR1/FF390 in real-time Q-PCR conditions, which was the case. BD: lower than detection threshold.

(DOC)

(DOC)

Table S5 Glomeromycota amplification on *Medicago truncatula* rhizosphere DNA extracts by real time Q-PCR in combination with FR1/FF390 primer set.

(DOC)

Table S6 Real-Time Q-PCR amplification results for the 5 soil samples used to test the specificity for fungi of FR1/FF390 primer set and to set up the template quantity in the real-time Q-PCR assay. NAN: Not A Number.

(DOC)

Table S7 Real-Time Q-PCR amplification results for the 24 soil samples used for the ecological validation of real-time Q PCR in combination with FR1/FF390 primer set. NAN: Not A Number.

(DOC)

Data S1 *In silico* analysis of literature primers and primer set selection. Sheet 01_Primers_List. List of the primers tested in the *in silico* analysis. Sheet 02_Primer_selection. Individual evaluation of each primer and primer selection results. Each primer was evaluated individually for its yield for fungi with 0, 1, 2 and 3 mismatches. A subset of "good" primers was selected according to the ratio between the number of sequences matched at k = 2 and k = 0 which measured whether the primer detected significantly more fungal sequences with two mismatches than with no mismatch. A well designed primer was therefore a primer that has a small ratio k2/k0 (threshold set at 1.2), because it cannot be improved using more degeneracies. Primers with large ratio k2/k0 were discarded from

the following analyses. A good primer is a primer that binds with a high percentage to every fungal clade but to a much lower extent to non fungal clades. **Sheet 03 Selected sets. Primer sets evaluation for the length of the amplicon produced by PCR.** A subset of primer sets was selected according to the length of the amplicon produced. The selection criterion was a short amplicon, shorter than the threshold limit determined by the length of the amplicon produced by primer set nu-SSU-0817–nu-SSU-1196. **Sheet 04.1 Sets evaluation. Fungal specificity and coverage evaluation of each primer set with exact match.** Data presented are the hit frequency (%) of each primer set for each phyla. **Sheet 04.2 Sets evaluation HAC. Raw data for hierarchical ascendant classification analysis of the primer sets. Data presented are a number of matched sequences. The first row indicates the names of the different phyla matched by the primer sets.** Number into brackets represent the total number of sequences per phyla. The results of the hierarchical ascendant classification analysis are presented in Figure S2. (XLS)

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Author Contributions

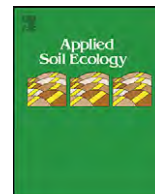
Conceived and designed the experiments: NCP-B SD LR. Performed the experiments: NCP-B SD LR ML. Analyzed the data: NCP-B RC LG CM. Contributed reagents/materials/analysis tools: CJ DA HRS. Wrote the paper: NCP-B RC CM LG LR.

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Seasonal dynamics of the bacterial community in forest soils under different quantities of leaf litter

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ABSTRACT

Soil microbial communities play an important role in soil carbon functioning, particularly in forest ecosystems. Their variation in response to climate change may affect soil carbon processes, highlighting the importance of understanding how environmental factors affect microbial communities. This study aimed to determine to what extent an increase in the quantity of fresh litter may affect heterotrophic mineralization of organic carbon and bacterial community structure in soil and litter. A litter manipulation experiment was performed *in situ* in a temperate deciduous forest. Three treatments of fresh litter inputs were considered: litter exclusion, natural conditions (control) and litter addition (twice the natural rate). Microbial and functional ecological approaches were combined to consider bacterial community structure in soil and litter using a molecular fingerprinting technique, and measurement of soil respiration both in terms of efflux intensity and isotopic composition of respired CO₂ (natural abundance) over one year.

The quantity of fresh litter seemed to affect soil and litter bacterial community structure and to interact with soil temperature and moisture to determine the temporal variation in the bacterial community on a month to season scale. In addition, this study highlighted the large temporal variability in soil and litter bacterial community structure and that this variability may affect our ability to relate bacterial community structure to respiration processes. This highlights the need for an intensive characterisation of the bacterial community structure to relate its variations to variations in soil respiration processes.

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1. Introduction

Terrestrial ecosystems play a crucial role in the global carbon cycle (Schlesinger and Andrews, 2000). In forest ecosystems, soil is of key importance in determining the carbon sink or source status of the ecosystem, because it is the largest stock of old carbon: ca. 70% of the carbon stock (Schlesinger and Andrews, 2000); and a large CO₂ source: 40–70% of the total ecosystem respiration (Janssens et al., 2001; Chambers et al., 2004). But in the context of climate change, soil carbon cycling is likely to be modified. According to the different *scenarii*, ecosystem productivity is predicted to increase with atmospheric CO₂ concentration (Norby et al., 2004; Handa et al., 2006; IPCC, 2007), leading to an increase in leaf biomass, leaf carbon:nitrogen ratio, and thus carbon allocation to soils (King

et al., 2004; Liu et al., 2005). In forests, this may strongly influence soil respiration by accelerating newly allocated carbon turnover (Lichter et al., 2005; Hoosbeek et al., 2007), in parallel to potential priming effects in soil (Sulzman et al., 2005; Fontaine et al., 2007; Nottingham et al., 2009) through variations in microbial community composition (Fontaine et al., 2003; Nottingham et al., 2009).

Microbial community composition plays a crucial role in determining the diversity of carbon mineralization processes and their stability in response to stress (Ramsey et al., 2005; Schimel et al., 2007; Nottingham et al., 2009). Indeed, *in situ* studies (transplantation experiments or environmental experiments; Reed and Martiny, 2007) showed that variations in soil microbial community were related to variations in the rates of different soil processes involved in carbon and nitrogen cycling (Waldrop et al., 2000; Balser and Firestone, 2005; Reed and Martiny, 2007). The functional significance of microbial community composition needs to be documented in the context of climate change, with particular reference to how variations in environmental factors determined by climate change affect soil microbial community structure and, consequently, soil functioning. This would help to better understand

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soil process modification and variability in response to variations in environmental factors and to facilitate the modelling of these processes (Davidson and Janssens, 2006), which is crucial in the context of climate change (Reed and Martiny, 2007).

Numerous studies have identified environmental factors affecting soil microbial community composition, at different spatial scales: from the ecosystem level to a 1 m range (Myers et al., 2001; Wilkinson and Anderson, 2001; Fierer et al., 2003; Lejon et al., 2005; Dequiedt et al., 2009); and different temporal scales: seasonal to monthly (Myers et al., 2001; Rogers and Tate, 2001; Fierer et al., 2003; Medeiros et al., 2006). These variations were mainly related to dominant plant species (Myers et al., 2001; Lejon et al., 2005), soil characteristics such as pH and texture (Fierer and Jackson, 2006), organic matter amount and quality (Bardgett et al., 1997; Lejon et al., 2007), and climatic factors such as the frequency of rainfall events (Fierer et al., 2003; Drenovsky et al., 2010). These factors have also been shown to induce variations in soil processes that were linked to modifications in the composition of the soil microbial community. Following soil transplantation, strong variation in soil temperature and moisture were found to affect carbon and nitrogen cycling by modifying the soil microbial community (Balsler and Firestone, 2005; Waldrop and Firestone, 2006), and changes in land use affected carbon cycling (Waldrop et al., 2000). But climate change is also predicted to increase the primary productivity of terrestrial ecosystems, increasing carbon allocation to soil (De Lucia et al., 1999; King et al., 2004; Liu et al., 2005; Crow et al., 2009). To explore the consequences of increasing carbon allocation to soil in the context of climate change, many studies took advantage of free air CO₂ enrichment (FACE) experiments (Phillips et al., 2002; Carney et al., 2007; Allison et al., 2007, 2008; Lipson et al., 2006) and highlighted significant modifications in the soil microbial community and soil processes. However, in these experiments, variations in carbon allocation to soil were mainly determined by litter fall and root biomass/exudation and the relative impact of these two carbon sources remains to be discussed. Litter-manipulation experiments mimic the increase in litter fall observed during FACE experiments, and therefore may offer a complementary strategy to study the impact of varying carbon allocation to soil on the microbial community, and its consequences for soil carbon functioning. Using this method, Rinnan et al. (2008) showed that soil microbial community composition of a subarctic heath soil was modified after fresh litter addition.

In this study, we performed *in situ* a litter-manipulation experiment in a temperate deciduous forest to determine to what extent an increase in fresh litter amount affects total soil respiration, heterotrophic mineralization of organic carbon and the bacterial community in soil and litter. Manipulation consisted of the reallocation of fresh litter to experimental plots according to three treatments: litter exclusion (E); control (C), *i.e.* natural fresh litter amount; and litter addition, *i.e.* at twice the natural amount (A). CO₂ efflux intensity was used as a measure of C mineralization activity in total soil and of microbial activity in bulk soil (without roots) and litter through *in situ* measurements and laboratory incubations (bulk soil and litter), respectively. Fresh litter reallocated to the plots was naturally significantly ¹³C depleted compared with soil (−1.8‰, Chemidlin Prévost-Bouré et al., 2010). Therefore, the isotopic composition ($\delta^{13}\text{C}$) of respired CO₂ was used to assess potential modifications in respiration substrates following litter allocation. The results relative to these measurements are detailed in a related paper (Chemidlin Prévost-Bouré et al., 2010). Briefly, total soil respiration (F_S) showed a significant increase with the quantity of fresh litter. The variation in F_S in the A treatment was related to “priming effect” processes in soil accounting for 32% of F_S on average (Chemidlin Prévost-Bouré et al., 2010). Added to that, despite the absence of variation in $\delta^{13}\text{C}$ of respired CO₂ from soil or litter following litter-manipulation, it experienced significant tem-

poral variation during the experiment. In view of these significant effects, it was considered useful to evaluate changes in soil and litter bacterial community structure in response to variation in leaf litter quantity, and to investigate the potential linkage of bacterial community structure variations with changes in respiration. Studying this link is “a substantial scientific challenge” according to Fitter et al. (2005) that may be achieved by coupling microbial ecology and functional ecology approaches.

In the present paper, the objective was to determine if these modifications of leaf litter amount affected bacterial community structure in soil or litter, and if these potential variations could be related to variation in soil respiration. To answer these questions, analyses of bacterial community structure in soil and litter were performed concomitantly with *in situ* and laboratory measurements of CO₂ efflux intensity and carbon isotopic composition. Measurements of bacterial community structure were performed using an automated DNA fingerprinting method (ARISA, Ranjard et al., 2001) directly applied on DNA extracted from soil and decomposing leaf litter. Then, measurements of bacterial community structure were related to CO₂ efflux intensity and isotopic composition in a co-inertia analysis to explore the link between bacterial community structure and soil functioning.

2. Methods

2.1. Site description

The experiment was performed in the Barbeau national forest (CARBOEUROPE IP network site – cluster.FR1, site FR-Fon; 48°29'N, 02°47'E; 60 km South-East of Paris, France; 90 m elevation). This is a managed mature oak forest (*Quercus petraea* L., 100–150 years old) with a dense understorey of coppiced hornbeam (*Carpinus betulus* L.). The climate is modified temperate maritime. The soil is a gleyic luvisol (FAO-ISRIC-ISSS 1998) of 0.8 m depth on millstone bedrock and the humus is an oligomull. Soil pH measured in water ranges from 4.5 to 5.4 and soil carbon content ranges from 0.2% to 14% of soil dry mass (decreasing with depth).

2.2. Experimental design

A 17.6 m² experimental area representative of the stand was chosen. From mid-October to the end of December 2005, fresh litter was collected from the experimental area with a net (0.5 mm mesh) installed 50 cm above the soil surface. Lateral litter inputs were prevented by completely surrounding the experimental area with another net directly attached to the posts supporting the collection net (0.5 mm mesh, 50 cm height). The collected fresh litter was air dried at ambient temperature and species composition was determined. It comprised oak (70% of total dry mass) and hornbeam (30% of total dry mass).

The experimental area was divided into 12 plots (each 1.2 m × 1.2 m) delimited by a PVC fence of 20 cm height. The plots were distributed following a randomised-complete-blocks design arranged into four blocks, each composed of three plots with one treatment each. Once before starting the experiment, the homogeneity of soil conditions among plots was checked for multiple parameters. The results are described in Chemidlin Prévost-Bouré et al. (2010).

In each block, three treatments were applied: fresh litter exclusion (E), control (C, 486 g m⁻² litter dry mass) and fresh litter addition (A, 972 g m⁻² litter dry mass). Litter was applied in the ratio of 70% oak to 30% hornbeam. Fresh litter collected during fall 2005 was deposited on 24 March 2006. During litter fall of autumn 2006, new litter inputs were avoided by placing the collecting nets upon and around the experimental area.

Monthly measurements of soil water content (0–10 cm depth, annual mean: 15.4% in the E treatment and in the control, and 15.3% in the A treatment) and temperature (10 cm depth, annual mean: 11.4 °C in every treatment, measurements performed between 9 am and 4 pm) showed that the soil temperature and water content were not affected by leaf litter quantity (Chemidlin Prévost-Bouré et al., 2010), although monthly measurement might not have captured the entire dynamics of soil temperature and moisture. However, litter bag studies also demonstrated that leaf litter degradation was not affected by the quantity of fresh litter (degradation coefficients: 2.01×10^{-3} and 1.93×10^{-3} , in the C and A treatments, respectively, Chemidlin Prévost-Bouré et al., 2010).

2.3. Soil and litter sample collection

Soil and litter samples were collected monthly during one year from all the treatment plots.

Three soil cores (0–10 cm depth, 1.2 cm diameter) were randomly taken from each plot, combined and sieved at 2 mm in the field to give a sample of ca. 50 g equivalent dry mass of soil. As the homogeneity between plots had been verified for pedological characteristics and soil respiration (Chemidlin Prévost-Bouré et al., 2010), a composite soil sample was obtained for each treatment by mixing subsamples from the four plots for a treatment in equal proportions.

Each month and for each plot, three litter samples were taken at random and combined. After lyophilisation (Christ alpha 2–4, Bioblock Scientific, Germany) and grinding in liquid N₂, a composite litter sample was obtained for each treatment by mixing subsamples from the four plots in equal proportions.

Finally, every composite sample was divided into three subsamples that were analyzed independently. The compositing approach was chosen according to Leckie et al. (2004) who demonstrated that composite sampling was a suitable method to characterize the genetic structure of microbial communities, limiting between-plot variability that can hamper the analyses of the DNA fingerprint. This approach was necessary to reduce intra-treatment variability and to increase the representativity of microbial community profiling (Schwarzenbach et al., 2007).

2.4. Bacterial community structure analysis

2.4.1. Extraction and purification of total DNA from soil and litter samples

For each sampling date and treatment, three subsamples weighting 1.3 g each were taken from the composite soil sample, and three subsamples weighting 0.5 g each were taken from the composite litter sample. All subsamples were analyzed independently.

Then, DNA extraction was performed following the procedure described in Ranjard et al. (2003) for both soil and litter samples, using a sample: extraction buffer ratio of 4:1 (v/w). DNA concentration ([DNA]) of crude extracts was determined as described in Ranjard et al. (2003) using a calf thymus standard curve. Crude DNA concentration ([DNA]) was used as an estimate of microbial biomass in soil and litter. Indeed, several studies have demonstrated a highly positive linear relationship between DNA recovery and C-biomass measurement (Chloroform Fumigation Extraction method), indicative of the size of the microbial biomass, in agricultural (Marstorp et al., 2000; Widmer et al., 2006; Bouzaiane et al., 2007) and forest soils (Bundt et al., 2001; Blagodatskaya et al., 2003), and to a lesser extent in organic layers of humus (Leckie et al., 2004).

Purification of crude DNA extracts was also performed as described in Ranjard et al. (2003) using PVPP (polyvinyl polypyrrolidone) minicolumns (BIORAD, Marne la Coquette, France) and

Geneclean Turbo kit (Q Biogene®, France) following the recommendations of the manufacturer.

2.4.2. Automated RISA fingerprinting

The Bacterial Automated Ribosomal Intergenic Spacer Analysis (B-ARISA) technique is a molecular fingerprinting method (Ranjard et al., 2000) that takes advantage of the length polymorphism of bacterial ribosomal InterGenic Spacer (IGS) and of the relative abundance of the different IGS sizes to characterize the genetic structure of soil bacterial communities.

The bacterial ribosomal IGS was amplified using the PCR protocol described in Ranjard et al. (2000). 25 ng of DNA was used as the template for PCR volumes of 25 µl. PCR products were purified using the MinElute Kit (QIAGEN, Courtaboeuf, France) and quantified using a calf thymus DNA standard curve. 2 µL of the product was added to deionized formamide and denatured at 90 °C for 2 min. B-ARISA fragments were resolved on 3.7% polyacrylamide gels under denaturing conditions as described in Lejon et al. (2005) on a LiCor® DNA sequencer (ScienceTec). The data were analyzed using the 1D-Scan software (ScienceTec), converting fluorescence data into electrophoregrams, where peaks represented PCR fragments. The height of the peaks was calculated in conjunction with the median filter option and the Gaussian integration in 1D-Scan, and represented the relative proportion of the fragments in the total products. Lengths (in base pairs) were calculated by using a size standard with bands ranging from 200 to 1659 bp.

2.5. Statistical analysis

Since the three subsamples derived from each treatment were analyzed independently for both microbial biomass and bacterial community structure, they were treated as independent replicates (see Section 2.3). In addition, the availability of data from 11 independent samplings ensured a robust data set for analysis.

The effect of the treatments on microbial biomass estimated by means of [DNA] was tested through non-parametric paired tests (Friedman repeated measures one-way analysis of variance on ranks, cited as RMANOVA; and the Kruskal–Wallis test). The significance level was set at 5% and the test was performed with Statistica software (Statsoft Inc, Tulsa, USA).

Data obtained from the 1D-Scan software were converted into a table summarizing the band presence (peak) and intensity (Gaussian area of peak) using the PrepRISA program (Ranjard et al., 2001); each row corresponded to a sample and each column corresponded to a band detected at least in one sample. 100 bands were integrated for each B-ARISA profile with a 2 bp resolution to ensure a robust analysis (Ranjard et al., 2003). The data were then analyzed through a principal component analysis (PCA) performed on the covariance matrix of the samples. Convex hulls were drawn over replicates of a treatment and the discrimination of the samples according to the treatment or the sampling period was tested through a Monte Carlo permutation test.

Euclidean distances derived from sample profiles were used as a measure of the magnitude of bacterial genetic structure modification between two treatments and calculated as described in Ranjard et al. (2008). The significance of this modification was tested using a Monte Carlo permutation test with at least 1000 permutations.

Soil functional factors (CO₂ efflux intensity and δ¹³C averages ± one standard error data), resulting from *in situ* measurements (total soil respiration) and laboratory measurements (bulk soil and litter incubations), were organized in a data matrix which was subjected to a principal component analysis (PCA) of

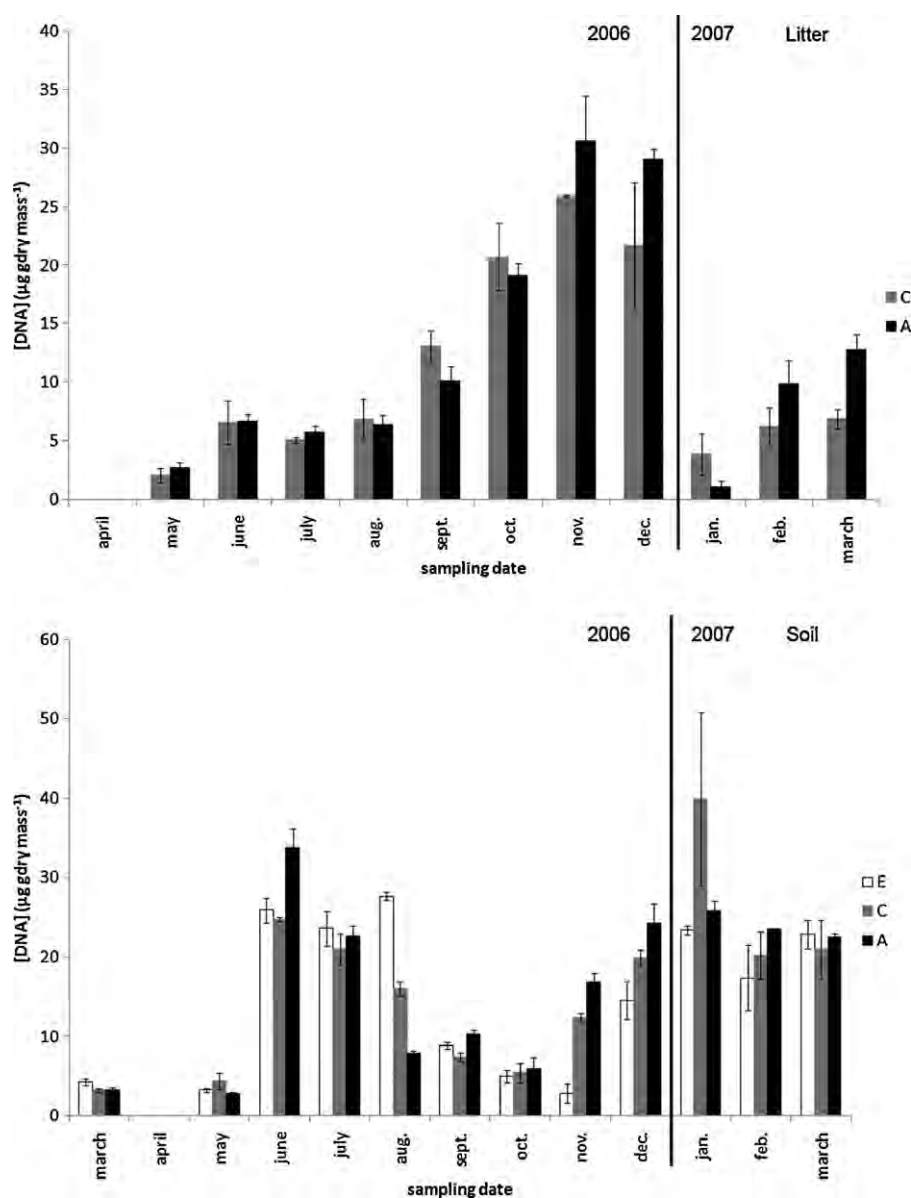


Fig. 1. Temporal variation in soil microbial biomass estimated by means of crude DNA concentration ([DNA], expressed in $\mu\text{g gdry mass}^{-1}$) in litter and soil in each treatment: E (litter exclusion, white bars), C (control, grey bars) and A (litter addition, black bars). Error bars represent standard error of the mean.

the correlation matrix. A co-inertia analysis (Dray et al., 2003) was used to study the relationships between the genetic structure of the bacterial communities and the set of functional factors. Co-inertia is a symmetric multivariate method used for coupling two or more data sets. It is based on the co-inertia criterion (also used in canonical correspondence analyses) and aims at maximizing the concordance (co-structure) between the data sets. A randomisation test of 1000 permutations was carried out to check the significance of the co-structure between the data sets (Monte Carlo test). When the co-structure between B-ARISA and soil functioning factors was significant, the corresponding correlation circle of the factors was drawn and superimposed on the B-ARISA factorial map to highlight the relationship between the CO_2 efflux intensity or $\delta^{13}\text{C}$ averages and the microbial community structure among the samples.

All statistical analysis on molecular fingerprints was performed using the ADE-4 software (Thioulouse et al., 1997).

3. Results

3.1. Dynamics of soil microbial biomass

Fig. 1a and b presents the temporal variations in [DNA] in litter and soil, respectively. The RMANOVA test showed that [DNA] was not affected by the quantity of fresh litter either in litter ($P > 0.6$), or in soil ($P > 0.1$). In both soil and litter, the seasonal trend for [DNA] was similar in each treatment, with significant variation over the course of the experiment ($P < 0.01$). Temporal variation in [DNA] in soil and litter were different from one another in both the C and A treatments. In soil, [DNA] increased from June to August, then decreased until early November and increased again at the end of the experiment. By contrast, in litter, [DNA] increased from September to the end of December and then decreased.

3.2. Dynamics of soil and litter bacterial community structure

Complex B-ARISA profiles were obtained in soil and litter and visual comparisons of profiles highlighted differences between soils sampled at different periods (Supplemental Fig. S1).

The results of Principal Component Analysis (PCA) of soil B-ARISA profiles obtained for each date in each treatment are presented in Fig. 2. PCA analysis of the soil bacterial community structure confirmed visual observations; with the different sampling periods distinctly discriminated along the first principal axis (PC1), that accounted for at least 29% of total variability, and much less so along PC2, that accounted for at least 14.0% of total variability. A permutation test confirmed that time was an important factor in discriminating soil bacterial community structure (BCS) both on PC1 and PC2. In the C and A treatments, conversely to the E treatment, July 2006 samples were clearly discriminated from the others along PC2. In addition, the Monte Carlo test distinguished between a warm period (from May 2006 to October 2006, $T_{\text{soil}} > 10^{\circ}\text{C}$) and a cold period (from November 2006 to March 2007, $T_{\text{soil}} < 10^{\circ}\text{C}$) in the E and C treatments. These two periods were not distinct in the A treatment. Temperature and moisture seemed to be important factors in determining the variations in bacterial community structure, particularly on PC2 in the control and A treatments, and on PC1 in the E treatment.

PCA analysis of litter B-ARISA profiles (Fig. 3) showed that the sampling dates were well distributed along PC2 which accounted for 24.6% and 16.4% of total variability in the C and A treatments, respectively. In both cases the convex hulls did not overlap, indicating temporal variation in litter bacterial community structure at a monthly time scale. Such PCA discrimination allowed the distinction of a cold and a warm period, along PC2. The period of soil drought (July 2006) was not discriminated from the other sampling dates in the analysis.

3.3. Effect of treatment on the structure of the bacterial community

Euclidean distances derived from the sample profiles were used as an estimate of the magnitude of genetic structure modification in the E and A treatments relative to the control; and between the E and A treatments. This method was applied to both soil and litter data.

Differences were observed in soil bacterial communities between E treatment and treatments C and A in May, June, and August 2006, and in February and March 2007 (Fig. 4a). The E treatment was also different from the A treatment in October 2006. Litter quantity also affected soil BCS as differences were observed between control and A treatment in May and October 2006, and in February and March 2007. On the other hand, no differences were observed between treatments in July and September 2006, and from November 2006 to January 2007.

When considering litter bacterial communities in C and A treatments per date, no effect of litter treatment on litter BCS was observed until December 2006. Differences between treatments were also observed in January and March 2007.

3.4. Relationships between the bacterial community structure and soil functioning

As soil and litter bacterial community structure changed among treatments and over time, the relationships between the bacterial community structure data and the corresponding soil functioning data presented in Chemidlin Prévost-Bouré et al. (2010) were investigated by co-inertia analysis. In bulk soil, the co-inertia analysis did not reveal any significant correlation between variation in

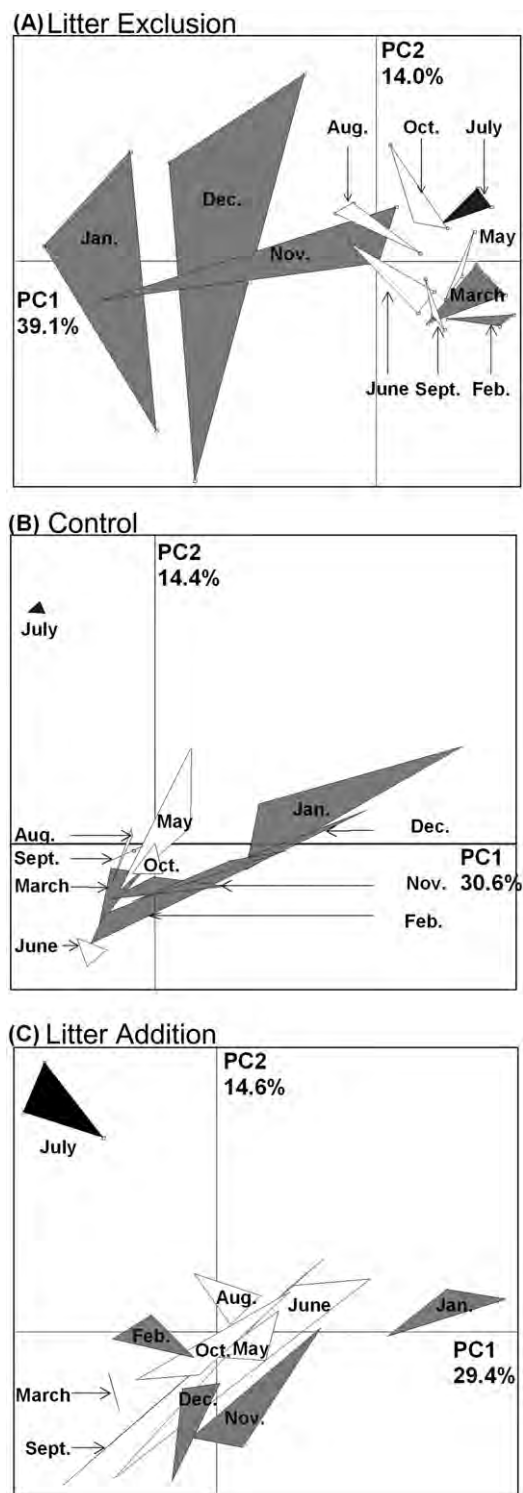


Fig. 2. Principal component analysis representation of the temporal variation in soil bacterial community under litter exclusion (A), Control (B) and litter addition (C) treatments. Samples were taken monthly without interruption from May 2006 until March 2007. Sampling periods when mean soil temperature at 10 cm depth was higher than 10°C are represented in white and those when mean soil temperature was lower than 10°C are represented in grey. July 2006, when the soil temperature was at a maximum and the soil water content was below the theoretical wilting point, is represented in black.

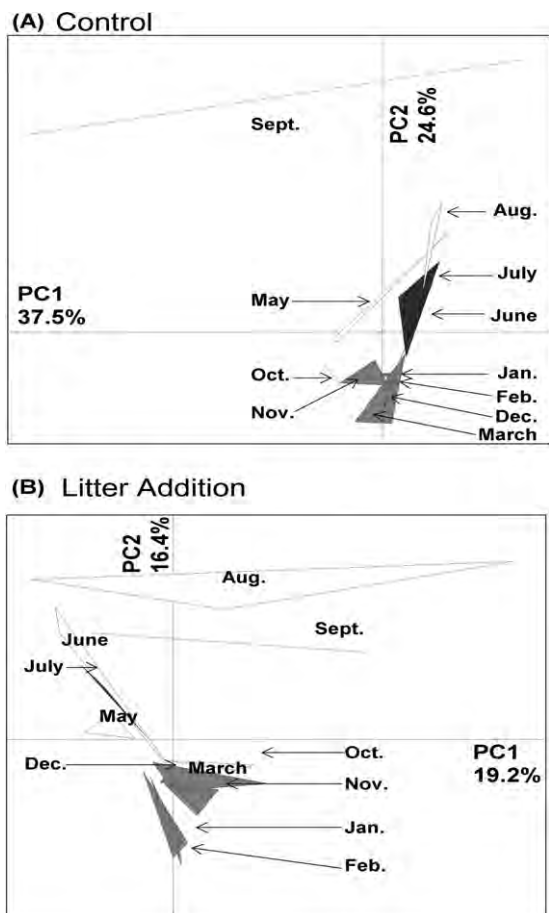


Fig. 3. Principal component analysis representation of the temporal variation in litter bacterial community in control (B) and litter addition (C) treatments. Samples were taken monthly without interruption from May 2006 until March 2007. Sampling periods when mean soil temperature at 10 cm depth was higher than 10 °C are represented in white and those when mean soil temperature was lower than 10 °C are represented in grey. July 2006, when the soil temperature was at a maximum and the soil water content was below the theoretical wilting point, is represented in black.

the bacterial community structure and the soil functioning factors. Contrastingly, in litter, the Monte Carlo test revealed a significant co-structure between litter bacterial community structure and functioning factors in the A treatment ($D=0.009$). In this case, sampling dates were distributed along PC1 (54%) and PC2 (25%) as observed in Fig. 5 and the co-structure was observed between litter bacterial community structure and litter CO_2 efflux intensity (F_L) and carbon stable isotopes composition ($\delta^{13}\text{C}_{F_L}$) measured in the laboratory. By contrast, total soil CO_2 efflux intensity (F_S) and carbon stable isotopes composition ($\delta^{13}\text{C}_{F_S}$) measured *in situ* did not seem to be related to litter BCS. No co-structure was observed in the control ($D=0.137$).

4. Discussion

Soil microbial communities are a crucial component of carbon cycling in forest ecosystems and their response to climate change could affect soil carbon processes (Davidson and Janssens, 2006). Therefore, it is important to better understand how environmental factors affect microbial communities. In Chemidlin Prévost-Bouré et al. (2010), we have demonstrated that increasing the quantity of fresh litter significantly increased the intensity of total soil CO_2 efflux (F_S) which was 1.9 times higher in the A treatment than in the Control. This increase in F_S was related to a priming effect in

the A treatment (32% of total soil CO_2 efflux measured *in situ*, estimated according to the method presented in Crow et al. (2009), in agreement with the definition provided by Bingeman et al. (1953) and updated by Kuzyakov (2010). Intensity of priming effect has previously been related to variations in microbial community composition through *in vitro* experiments (Bernard et al., 2007) and field experiments (Carney et al., 2007), which may lead to variation in respired substrates that could affect the isotopic composition of CO_2 efflux from total soil, bulk soil or leaf litter. These observations highlight the need to evaluate variation in bacterial community structure in soil and litter, and to relate these data to soil respiration in order to better understand the link between soil biodiversity and functioning.

4.1. Seasonal variation in soil and litter microbial biomass

In this study, neither soil nor litter microbial biomass were affected by the quantity of fresh litter. This lack of variation may be due to the method used to measure microbial biomass that targets the whole microbial biomass and not only its active part which is commonly assumed to represent only a few percent of the total microbial biomass (Nannipieri et al., 2003). Therefore, litter-related variation in the ratio of active to total microbial biomass cannot be excluded. In all treatments, soil and litter microbial biomass exhibited seasonal variation, which is consistent with other studies performed in forest, grasslands, and agricultural plots (Wardle, 1998; Bardgett et al., 1999).

The high soil microbial biomass recorded in summer has been frequently observed in forests or tree-dominated ecosystems (Wardle, 1998; Jiang and Xu, 2006; Miller et al., 2009). However, the high levels of soil microbial biomass recorded in winter after a decline in autumn were surprising and may have resulted from microbial growth in winter in response to favourable conditions provided by the relatively high soil temperature (8.0 °C on average in winter 2007 compared to an annual mean of 11.4 °C), and high soil water content (20.4% on average for an estimated field capacity of 20.2%) increasing the accessibility to water soluble C, and potential fresh carbon inputs *via* root death, fresh carbon being a growth-limiting factor for soil microorganisms (Ekblad and Nordgren, 2002).

In litter, the seasonal variation in microbial biomass was different from that in soil. The maximum litter microbial biomass was observed in late autumn and could have resulted from the colonisation of litter by soil microorganisms.

4.2. Seasonal variation in soil and litter bacterial community structure

Soil and litter bacterial community structures varied with season with differences between a warm and a cold period being observed in all treatments except for soil in the A treatment, despite climatic conditions being identical in all treatments (Chemidlin Prévost-Bouré et al., 2010). Therefore, at the seasonal time scale, variation in bacterial community structure in soil or litter might be dependent on temperature (Bardgett et al., 1997; Myers et al., 2001; Rogers and Tate, 2001; Petersen et al., 2002; Buckley and Schmidt, 2003). At a monthly time scale, the absence of substantial overlap of samples in the PCA analysis suggests that the genetic structure of soil bacterial communities may experience rapid change, which may be partly determined by variation in soil moisture. Indeed, the water stress observed in the superficial soil layer (July 2006) was concomitant with a strong modification of soil bacterial community structure and many studies have highlighted the effect of soil moisture and rainfall frequency on soil bacterial community structure (Wilkinson et al., 2002; Fierer et al., 2003; Papatheodorou et al., 2004; Williamson and Wardle, 2007). In contrast, in litter,

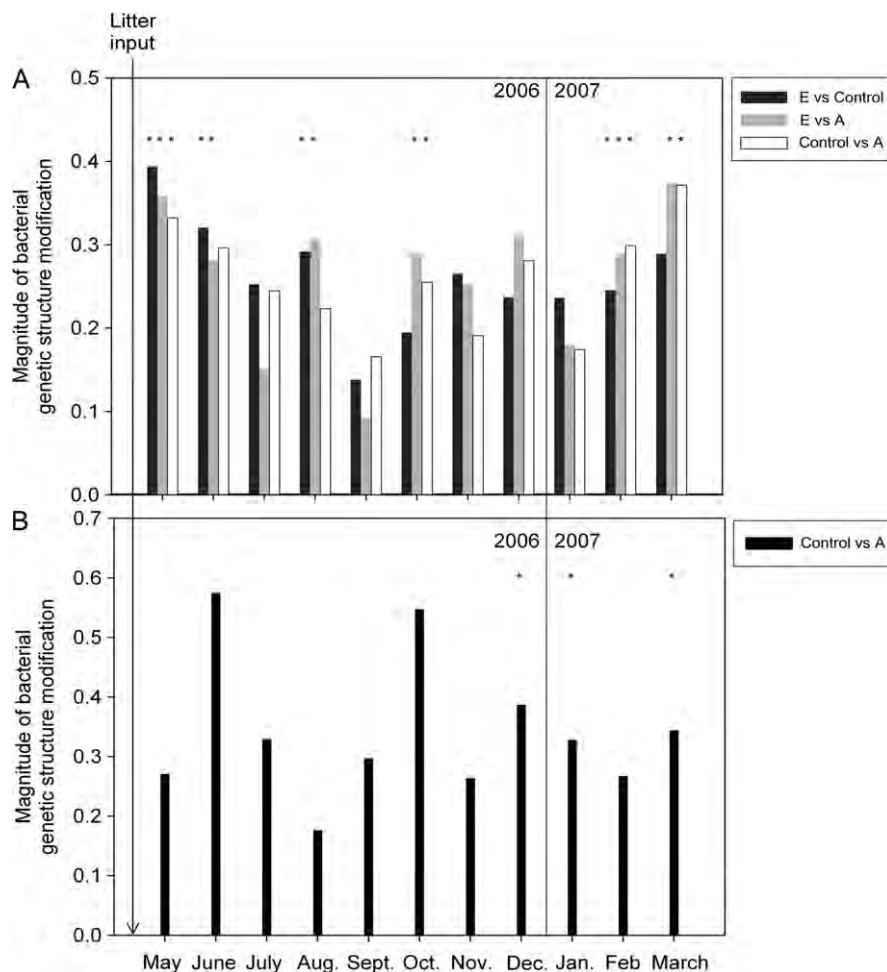


Fig. 4. Comparison in Euclidean distances of (A) soil bacterial community structure between the different litter treatments: E vs. control (black bars, reference: control), E vs. A (grey bars, reference: E), and control vs. A (white bars, reference: control), and (B) litter bacterial community structure in the control (reference) and the A treatment.

bacterial community structure was not affected by soil drought stress. The difference in response between soil and litter bacterial communities to water stress might be conceptually explained by a pre-adaptation process (Tobor-Kaplon et al., 2005; Bressan et al., 2008) in litter that is more frequently submitted to drying/rewetting cycles than soil, making litter microbial communities more resilient than soil microbial communities in response to this kind of perturbation.

The warm and cold periods in this study correspond respectively to a period of active photosynthesis in trees and exudation of labile carbon compounds (Ekblad and Hogberg, 2001), and to a period of highly reduced tree activity. During these periods, variation in tree physiology may induce changes in the quantity and/or composition of root exudates which are determinant for variation in soil bacterial community structure (Marschner et al., 2002). In litter, the literature suggests that seasonal variation in litter bacterial community structure is mainly driven by the changes in leaf litter biochemical composition during litter decomposition (Aneja et al., 2006; Nicolardot et al., 2007; Bastian et al., 2009). Primarily, easily degradable compounds (residual sugars and starch) are decomposed rapidly leading to an increase in the relative abundance of more complex molecules (cellulose) and recalcitrant material (lignin) in litter (Fernandez et al., 2003; Aneja et al., 2006), contributing to the selection of particular groups of microorganisms able to decompose recalcitrant materials. This trend may have been strengthened in our study by the experimental exclusion of lit-

ter in autumn 2007, avoiding the input of new easily degradable compounds in the experimental area at the beginning of the cold period.

4.3. Influence of litter quantity on variation in bacterial community structure in soil and litter

In litter, variation in the bacterial community structure was observed only at the end of the experiment during the “cold” period. As litter decomposition rates were the same, we may assume that increasing the quantity of litter may have affected the litter bacterial community by increasing the availability of ecological niches (physical or trophic; Ranjard and Richaume, 2001). However, as differences appeared only during the “cold” period, favourable temperatures may have buffered the impact of leaf litter amount on bacterial community structure during warmer periods.

In soil, nutrient availability might have differed between the plots where litter was excluded and the plots with litter (natural amount or double amount) because of nutrient leaching from the litter compartment to the soil (Gaillard et al., 1999; Park and Matzner, 2003). This may have selectively affected different groups of soil microorganisms between litter exclusion plots and plots with litter. In both C and A treatments, the litter degradation rate was the same and litter microbial biomass was not affected either, leading to the assumption that the release of molecular compounds from litter to soil was larger in the A than in the C treatment, stimulating under-represented soil microorganisms in the A treatment

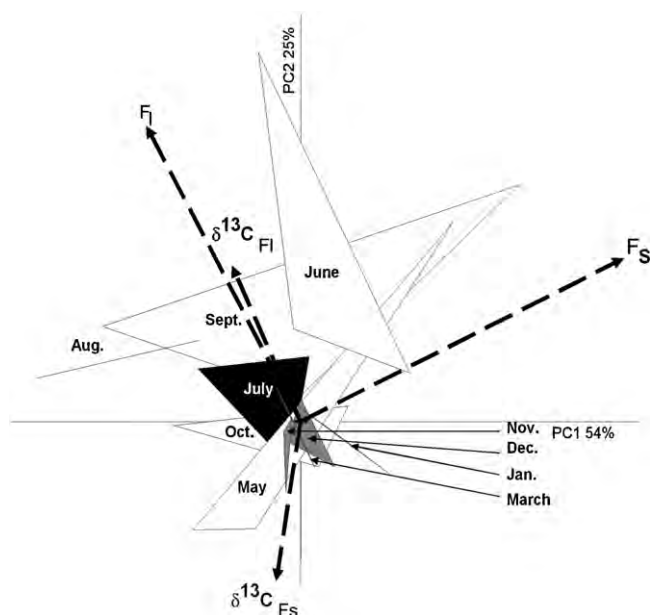


Fig. 5. Co-inertia analysis of the relationships between the structure of the litter bacterial community and CO₂ efflux intensity (F_S) and the carbon isotopic composition ($\delta^{13}C_{F_S}$) of litter in the A treatment. Sampling periods when mean soil temperature at 10 cm depth was higher than 10 °C are represented in white and those when mean soil temperature was lower than 10 °C are represented in grey. July 2006, when the soil temperature was at a maximum and the soil water content was below the theoretical wilting point, is represented in black. Arrows represent the contribution of each functional variable (F_S , F_L , $\delta^{13}C_{F_S}$ and $\delta^{13}C_{F_L}$) to the discrimination of the bacterial community structure of the different litter samples in the co-inertia analysis.

(Marschner et al., 2002; Aneja et al., 2006; Williamson and Wardle, 2007). This hypothesis is in agreement with the long-lasting priming effect recorded in the A treatment (representing 32% of total soil CO₂ efflux intensity; Chemidlin Prévost-Bouré et al., 2010) and with the conceptual model of Fontaine et al. (2003). Indeed, higher nutrient leaching in the A treatment may have: (i) stimulated *r*-strategist growth which may in turn stimulate *K*-strategist growth according to mechanism 1 of Fontaine et al. (2003), or (ii) decreased the competition between *r*- and *K*-strategist populations, allowing *K*-strategist growth and the consumption of soil organic matter according to mechanism 2 of Fontaine et al. (2003). However, this does not preclude the intervention of roots in the priming effect processes as suggested in Chemidlin Prévost-Bouré et al. (2010) and proposed by Kuzyakov (2010). Further investigation is needed to validate these hypotheses.

Additionally, the leaf litter amount may have modulated the impact of climatic factors in determining the amplitude of soil bacterial community structure variations, as soil bacterial community structure seemed to be affected by temperature only in the E treatment and in the Control at a seasonal time scale, and no differences in soil bacterial community structure were observed between the treatments during the drought stress period. Such interaction between litter amount and environmental factors has been recently reported for a dwarf shrub/graminoid heath in Sweden (Rinnan et al., 2008) and for a litter bag experiment (Aneja et al., 2006) and may have important consequences for the response of soil bacterial community structure to climate change.

4.4. Link between soil respiration and bacterial community structure

The link between microbial community composition and soil processes has been previously investigated through environmen-

tal studies and transplantation studies on carbon and/or nitrogen cycles (Waldrop et al., 2000; Balser and Firestone, 2005; Carney and Matson, 2005; Lipson et al., 2006; Reed and Martiny, 2007). In this environmental study, we attempted to relate temporal variations in bacterial community structure and CO₂ efflux characteristics (intensity, carbon stable isotope composition) in total soil, bulk soil and litter through a co-inertia analysis. Total soil CO₂ efflux intensity and isotopic composition were measured *in situ*, while bulk soil and litter CO₂ efflux intensities and isotopic compositions were determined by means of laboratory incubations (Chemidlin Prévost-Bouré et al., 2010).

According to the co-inertia analysis, the structure of the litter bacterial community seemed to be related to CO₂ efflux characteristics in the A treatment but not in the control. These different trends between the control and the A treatment may be explained by seasonal uncoupling of microbial community composition (Bell et al., 2009). Indeed, in this study, litter bacterial community structure tended to evolve differently between the control and the A treatment at the end of the experiment, highlighting potentially different responses to temperature and moisture.

When seasonal variations in litter bacterial community structure seemed related to the CO₂ efflux characteristics (A treatment), the link was mainly observed for litter CO₂ efflux intensity (F_L) and carbon stable isotope composition ($\delta^{13}C_{F_L}$) and not for total soil CO₂ efflux intensity (F_S) or carbon stable isotope composition ($\delta^{13}C_{F_S}$). This difference may be explained by the relatively small contribution of litter to total soil respiration in our system (2–18%, Chemidlin Prévost-Bouré et al., 2010), statistically uncoupling litter bacterial community structure from total soil CO₂ efflux characteristics.

In contrast to litter, soil bacterial community structure seemed to exhibit a weaker link with CO₂ efflux characteristics whatever the treatment was. According to Nicolardot et al. (2007), litter is much more selective than soil. Therefore, it may be assumed that functional redundancy is higher in soil than in litter, which would limit our ability to relate bacterial genetic structure and CO₂ efflux characteristics in soil. This limitation may have been enhanced by the relatively small proportion of active bacterial taxa in soil (Rogers and Tate, 2001; Dilly, 2006; Bernard et al., 2007; Blagodatskii et al., 2008).

In addition, other microorganisms may have played an important role in respiration processes, particularly fungi. This may have precluded linking soil bacterial community composition and CO₂ efflux characteristics. The importance of fungi in soil functioning, particularly in forests, has been recognized and well documented. Nevertheless, this study underlines the importance of bacteria in the degradation of organic matter in these soils.

5. Conclusion

This study provides evidence for the importance of litter amount and its interactions with abiotic factors (temperature and/or moisture) in determining the dynamics of soil and litter bacterial community structure in forest ecosystems, interactions that may influence the relationship between soil respiration processes and bacterial community structure. However, this needs to be confirmed through further experiments taking advantages of litter manipulation, soil transplant and labelling techniques to decipher the respective importance of biotic and abiotic factors in determining the dynamics of bacterial community structure and its link to soil functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apsoil.2010.11.006.

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Increase in aboveground fresh litter quantity over-stimulates soil respiration in a temperate deciduous forest

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ABSTRACT

In the context of climate change, the amount of carbon allocated to soil, particularly fresh litter, is predicted to increase with terrestrial ecosystem productivity, and may alter soil carbon storage capacities.

In this study we performed a 1-year litter-manipulation experiment to examine how soil CO₂ efflux was altered by the amount of fresh litter. Three treatments were applied: litter exclusion (E), control (C, natural amount: 486 g m⁻²) and litter addition (A, twice the natural amount: 972 g m⁻²).

Litter decomposition rate was not affected by fresh litter amount. However, the addition or exclusion of fresh litter quickly increased or decreased total soil CO₂ efflux (F_S) significantly, but the relative contribution of fresh litter to total soil respiration remained unchanged between the C and A treatments, as determined by laboratory measurements. Variation in F_S among treatments was not related to modification of its temperature sensitivity which was not affected by fresh litter amount (Q_{10} : 3.5 for E, 3.2 for C, 3.6 for A). While litter exclusion was the main cause of the F_S decrease in the E treatment, only 68% of F_S was directly attributable to litter addition in the A treatment. The remaining 32% of F_S in the A treatment was related to a real priming effect that appeared to be a long-lasting phenomenon. This priming effect lasting over 1 year may be related to a continuous release of organic compounds from litter to soil because of the progressive decomposition of leaf litter. Q_{10} estimates and isotopic data lead to the hypothesis that the priming effect corresponded to the activation of the whole soil system.

As a consequence, the increase in ecosystem productivity may lead, *via* an increase in the amount of litter, to an increase in carbon turnover in soil. Further labelling experiments involving high-frequency carbon stable isotope measurements of CO₂ efflux would help to clarify the relative importance of bulk soil and rhizosphere in the priming effect.

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1. Introduction

In the context of climate change, the importance of the litter compartment as a carbon source for total soil CO₂ efflux (F_S) will increase according to the predicted increase in litter fall with atmospheric CO₂ concentration (King et al., 2004; Liu et al., 2005; Crow et al., 2009). Several studies have investigated the contribution of the leaf litter layer to F_S by means of organic layer removal (Buchmann, 2000; Li et al., 2004) or carbon stable isotope tech-

niques (¹³C depleted litter, Ngao et al., 2005). In temperate forest ecosystems, the estimates of leaf litter contribution to F_S exhibit a large range of seasonal variation: from 2% to 20% of annual soil CO₂ efflux in both deciduous (Edwards and Sollins, 1973; Bowden et al., 1993; Ngao et al., 2005) and coniferous forests (Nakane et al., 1997; Buchmann, 2000).

The quantity of fresh litter remains an important factor affecting soil carbon cycling. Long-term litter-manipulation experiments performed *in situ* showed that an increase in the quantity of fresh litter led to an increase in F_S (Boone et al., 1998; Subke et al., 2004; Sulzman et al., 2005; Crow et al., 2009). Moreover, several laboratory studies have provided valuable evidence that variation in fresh organic carbon amount or quality may affect soil carbon cycling *via* "priming effect" processes: an increase in soil organic carbon mineralization following the input of fresh organic carbon residues

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(Bingeman et al., 1953; Kuzyakov et al., 2000; Nottingham et al., 2009); and *via* changes in diversity of organisms involved in soil functioning (Fontaine et al., 2004; Nottingham et al., 2009). These studies suggest that a priming effect commonly occurs in most plant–soil systems as frequent fresh organic residue inputs appear through litterfall or fine root death. Quantitative variations in fresh organic carbon inputs to soil (determined by climate change) are likely to rapidly affect the whole soil organic carbon mineralization process under natural conditions (Fontaine et al., 2004, 2007; Sulzman et al., 2005; Crow et al., 2009). Understanding how soil carbon cycling may be modified needs consideration of the response of heterotrophic respiration components (litter respiration and bulk soil respiration; referred to in the following as F_l and F_m , respectively) to quantitative variations of fresh organic carbon inputs.

In parallel with CO_2 efflux, measurements of the natural abundance of ^{13}C have been used to improve our knowledge of the origins of carbon pools used for soil respiration. In forest soils, clear temporal variation in CO_2 efflux $\delta^{13}\text{C}$ values (up to 3.5‰) has been observed (Ekblad et al., 2005; Högberg et al., 2005; Mortazavi et al., 2005; Marron et al., 2009) and correlated with variation in the contribution of the different respiration sources to total soil respiration and to shifts in the $\delta^{13}\text{C}$ values for respiration substrates (Ehleringer et al., 2000; Biasi et al., 2005; Högberg et al., 2005; Tu and Dawson, 2005; Kuzyakov, 2006). As a result, $\delta^{13}\text{C}$ measurements would help to examine whether the amount of litter induces a significant change in the respective contributions of the different soil components and/or respiration substrates mobilised by some “priming effect” process (Fontaine et al., 2007).

In this study, we performed an *in situ* litter-manipulation experiment over 1 year to determine to what extent an increase in the amount of fresh litter affects the heterotrophic mineralization of organic carbon in a temperate deciduous forest. Litter manipulation lasted 1 year and consisted in the allocation of fresh litter to experimental plots according to three treatments: litter exclusion (E); natural fresh litter amount, *i.e.* control (C); and litter addition at twice the natural amount (A). Every month, measurements of the rate and carbon isotopic composition ($\delta^{13}\text{C}$) of total soil CO_2 efflux were performed *in situ* (field measurements) and measurements of the rate and carbon isotopic composition ($\delta^{13}\text{C}$) of heterotrophic CO_2 efflux in litter and soil (without roots) were performed *in vitro* (short-term incubations, laboratory measurements). In parallel, soil and litter bacterial community structure were monitored by means of molecular fingerprinting. “Functional” data (CO_2 efflux and carbon isotope composition measurements) are presented in this manuscript. Data concerning the dynamics of microbial communities are presented by Chemidlin Prévost-Bouré et al. (*in press*) where they are related to “functional” data to explore the link between bacterial community structure and soil functioning.

2. Materials and methods

2.1. Site description

The experiment took place in Barbeau National Forest (CARBOEUROPE IP network site [Cluster_FR1, Site_FR-Fon]; 48°29'N, 02°47'E; 60 km southeast of Paris, France; 90 m elevation). This site is a managed mature oak forest (*Quercus petraea* L., 100–150 years old) with a dense understorey of coppiced hornbeam (*Carpinus betulus* L.). The mean annual temperature was 10.7 °C and the annual rainfall was 680 mm (1980–1996).

The soil was a gleyic luvisol (IUSS Working Group WRB, 2006) of 0.8 m depth on millstone bedrock covered with an oligomull humus. The soil texture was loam in the top soil (USDA referential; Sand: 41.9%; Clay: 19.3%; Silt: 38.8%) and clay loam at the bottom of the soil profile (USDA referential; Sand: 35.2%; Clay: 32.8%;

Silt: 32.0%). The main soil characteristics were determined by the INRA Soil Analysis Laboratory at Arras (France). The granulometric fractions were determined according to the French norm NF X 31-107 by the dispersion of mineral particles after destruction of the organic matter by hydrogen peroxide and separation of the particles into different classes by sedimentation (particles <50 μm) and sieving (particles >50 μm; Gee and Bauder, 1986). Gleyic colour patterns were observed from 30 cm depth down to the bottom of the soil profile. The soil pH measured in water (NF ISO 10390, in a 1:5 (v/v) ratio of soil and water suspension) and the soil carbon content (in a 1 g sample calcinated at 550 °C and crushed to 250 μm) both decreased with depth. The soil carbon content was 14.5% of soil dry mass at the soil surface and 0.2% of soil dry mass at 10 cm depth.

2.2. Experimental design

A 17.6 m² experimental area (two 1.2 m × 7.3 m rows) representative of the stand was chosen. From mid-October to the end of December 2005, fresh litter was collected from the experimental area with a net (0.5 mm mesh) installed 50 cm above the soil surface. Lateral litter inputs were prevented by surrounding the experimental area with another net (0.5 mm mesh). The collected fresh litter was air-dried at ambient temperature and the species composition was determined as oak (70% of total dry mass) and hornbeam (30% of total dry mass).

The experimental area was divided into 12 adjoining plots (each 1.2 m × 1.2 m) delimited by a PVC fence of 20 cm height. Treatments were applied in the field following a randomised-complete-block design with one replicate of each treatment per block. Before starting the experiment, the homogeneity of the plots was checked once for: environmental conditions (soil water content, soil temperature at 10 cm depth); carbon content and isotopic composition of total soil organic carbon and “old” litter (naturally deposited during autumn 2005); F_S , F_m , $\delta^{13}\text{C}_{F_S}$ and $\delta^{13}\text{C}_{F_m}$. The carbon content and the isotopic composition of total soil organic carbon and remaining litter were determined using the GC-C-IRMS system described in Section 2.7. “Old” litter mass was estimated by sampling litter in the four corners of the plots (15 cm × 15 cm), drying at 50 °C and weighing.

The three treatments were: litter exclusion (E), natural conditions, *i.e.* control (C, 486 g m⁻² litter dry mass) and litter addition (A, 972 g m⁻² litter dry mass). Litter was added to treatments C and A on 24 March 2006 in the observed 7:3 oak:hornbeam ratio. During leaf fall in autumn 2006 new litter inputs were avoided by replacing the collecting nets upon and around the experimental area.

Every measurement described in the following was performed monthly from May 2006 to March 2007, except for litter mass loss which was measured every 2 months. Carbon isotopic composition is expressed in ‰ relative to the Pee Dee Belemnite standard.

2.3. Fresh litter degradation measurements

During litter deposition, 12 litter bags (mesh size: 1 mm) containing 5 g of litter ground by hand were placed in each plot of the C and A treatments. Three litter bags were collected every 2 months; their contents were dried at 50 °C for 48 h and weighed. Fresh litter mass loss was estimated by calculating the difference between the initial litter dry mass and the remaining litter dry mass. The fresh litter degradation coefficient (k) was estimated according to Granier et al. (2000) as the exponent of an exponential decay relationship between remaining litter dry mass (M_t) and time since deposition (t , days). M_0 is the initial litter dry mass:

$$M_t = M_0 \times e^{(-k \times t)} \quad (1)$$

2.4. Field measurement of total soil CO₂ efflux intensity: F_S

F_S was measured in the field on circular collars (12 cm diameter, 7 cm height, 4 collars per plot) buried 2 cm into the soil before litter addition. In each collar the litter mass per unit area was equivalent to that in the relevant plot. Four measurements were performed per plot (one per collar) and averaged. Manual F_S measurements were conducted on these collars monthly with a CIRAS analyser (PP Systems International Inc., Amherst, MA) configured in closed system with SRC-1 soil respiration chamber (wind speed = 0.1 m s⁻¹, as recommended by Le Dantec et al., 1999). Measurements lasted 2 min or the time needed for an increase in CO₂ concentration of 100 ppm in the chamber. Prior to measurements, the stability of fluxes was tested on two collars per plot by three repeated measurements. Mean F_S values were calculated for each plot, expressed in μmol m⁻² s⁻¹. Measurements were converted to gC m⁻² for comparison with fresh litter mass loss derived from the fresh litter degradation model.

During F_S measurements, the soil temperature was measured at 10 cm depth around each collar using a linear precision resistance probe (STP-1, PP Systems). The soil water content (SWC, %) was measured for the 0–10 cm layer by drying soil at 105 °C and relating the mass loss to soil dry mass.

2.5. The role of litter in the modification of total soil CO₂ efflux intensity

To determine the role of litter in the modification of total soil CO₂ efflux intensity, the CO₂ efflux intensity measured *in situ* was compared to the expected CO₂ efflux intensity calculated as follows:

$$F_{S,E}^{\text{EXPECTED}} = F_{S,C} - \text{LMM}_C \quad (2)$$

$$F_{S,A}^{\text{EXPECTED}} = F_{S,C} + \text{LMM}_C \quad (3)$$

where, in Eq. (2), F_{S,E}^{EXPECTED} is the expected flux in the E treatment calculated from the measured efflux in the control (F_{S,C}) minus the decay of litter (LMM_C) in the control, which corresponds to the litter excluded in the E treatment; and where, in Eq. (3), F_{S,A}^{EXPECTED} is the expected flux in the A treatment calculated from the measured efflux in the control (F_{S,C}) plus the decay of litter (LMM_C) in the control, which corresponds to the litter added in the A treatment. Every efflux was calculated in gC m⁻². These equations were previously described by Crow et al. (2009). If the expected efflux intensity is equivalent or higher than the measured efflux intensity, the difference in the efflux intensity between the treatment and the control may be attributed to the decomposition of litter. If the expected efflux intensity is lower than the measured efflux intensity, it would be assumed that litter decay cannot be the only source explaining the increase of efflux intensity and that this increase corresponds to soil priming according to Crow et al. (2009). In these conditions, soil priming (PE) would be determined as the percent of total soil CO₂ efflux measured *in situ* according to the equation described by Crow et al. (2009):

$$\text{PE} = 100 \times \left[\frac{F_S - F_S^{\text{EXPECTED}}}{F_S} \right] \quad (4)$$

where, for a given treatment, F_S corresponds to soil respiration measured *in situ* and F_S^{EXPECTED} is the expected soil respiration determined according to the equations described above (Eq. (2) or (3)).

2.6. Laboratory measurement of heterotrophic CO₂ efflux in litter and soil without roots: F_l and F_m

2.6.1. Sampling

Soil and litter samples were collected monthly in every plot according to the treatment. The soil sample consisted of three randomly taken soil cores (0–10 cm depth, 1.2 cm diameter) which were combined and sieved at 2 mm in the field (ca. 50 g equivalent dry mass of soil).

Litter samples were also composite samples constituted of three samples randomly taken in each C and A plots. Litter samples per plot corresponded to ca. 2.5 g equivalent dry mass of litter.

2.6.2. CO₂ efflux measurement

About 1.5 h after sampling, soil and litter samples were incubated in the laboratory using flasks sealed with a Teflon® septum. Measurements were carried out on three 5 g replicates per plot for soil and one replicate for litter because of limited material. At the beginning of the incubation, flask air was decarboxylated by pushing air several times through a soda-lime column. Then samples were incubated for 13–20 h to avoid strong variations in terms of microbial biomass and diversity, and to limit shifts in metabolic pathways or mineralized carbon sources (Andrews et al., 1999). Incubations were performed in a water bath regulated at the soil temperature previously recorded in the field. At the end of the incubation, 25 mL of flask air was sampled with a 50 mL valved syringe (SGE) and injected into new flasks flushed with pure nitrogen. Air samples were analysed for their CO₂ concentration and δ¹³C_{CO₂} (see Section 2.7 for details).

F_l and F_m were calculated as the ratio between CO₂ concentrations accumulated during the incubation over incubation time. To scale to field measurements, laboratory measurements of F_l were converted from μmol g⁻¹ dry mass s⁻¹ to μmol m⁻² s⁻¹ using litter dry mass present on the field plots. Litter dry mass on the field plots was determined as the difference between the initial litter dry mass and the litter mass lost during the elapsed time between the start of the experiment and the date of sampling as estimated via the exponential decay function derived from fresh litter degradation measurements. Then, F_l was used to estimate the contribution of fresh litter to total soil CO₂ efflux. F_m was also expressed in μmol m⁻² s⁻¹ using the apparent density of soil (1.35) and soil core volume (1.13 × 10⁻⁵ m³). Conversely to F_l measurements, F_m measurements were only used to test the impact of the treatment on heterotrophic soil CO₂ efflux intensity. Because sieving modified the conditions of diffusion, these measurements did not allow the estimation of the contribution of F_m to total soil CO₂ efflux.

2.7. Measurement of the isotopic composition of respired CO₂: δ¹³C_{F_S}, δ¹³C_{F_l} and δ¹³C_{F_m}

The isotopic ratio of total soil-respired CO₂ (δ¹³C_{F_S}) was determined in the field for each plot by the Keeling plot method (Keeling, 1958). Soil air was sampled in the field using a pump-equipped EGM-1 analyser (PP Systems) and a custom cylindrical closed chamber (25.4 L, 12 cm height) made of transparent acrylic resin and equipped with a fan. The chamber was laid on a collar inserted 2 cm into the soil. For each Keeling plot, five air samples were taken directly from the closed circuit using 50 mL valved syringes (SGE, Australia). Air samples were taken every 100–250 ppm CO₂ increase in the range 500–1500 ppm. Air samples were analysed for their δ¹³C_{CO₂} with an elemental analyser (Model NA-1500, Carlo Erba, Milan, Italy) coupled to an isotopic ratio mass spectrometer (VG Optima, Fison, Villeurbanne, France; measurement standard error = 0.2‰). Linearity and stability of the analysis system were tested during measurements by analysing standard air samples of

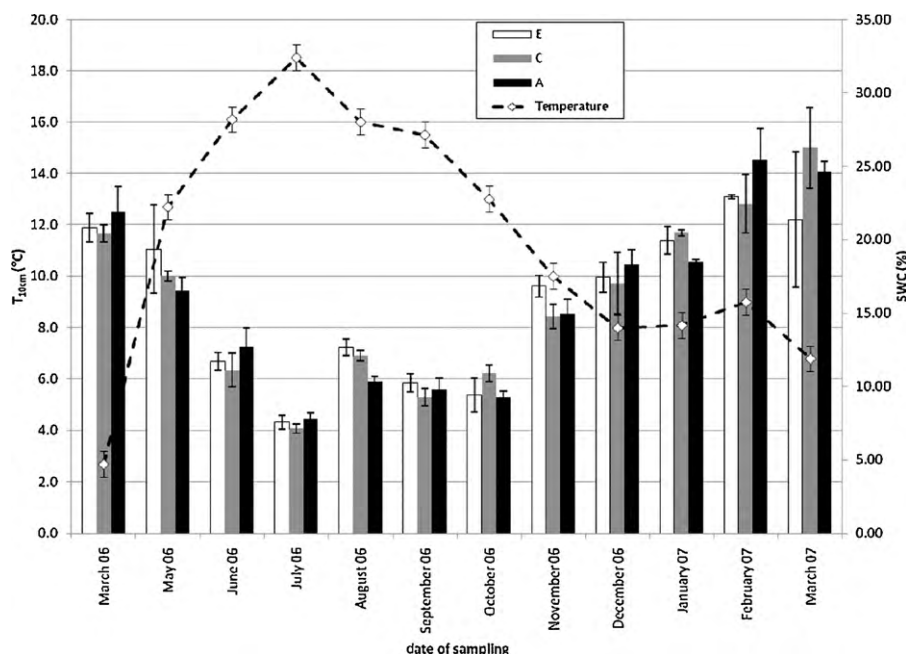


Fig. 1. Temporal variation in soil temperature at 10 cm depth ($T_{10\text{cm}}$, diamonds, dotted line) and soil water content (SWC, %, bars) in the three treatments: litter exclusion (E, white bars), control (C, grey bars) and litter addition (A, black bars). Error bars represent standard error of the mean ($n=3-4$).

different CO_2 concentration and with the same $\delta^{13}\text{C}$. $\delta^{13}\text{C}_{F_S}$ was estimated as the y -intercept of the linear regression between the inverse of CO_2 concentration (x -axis) and $\delta^{13}\text{C}_{\text{CO}_2}$ (y -axis). Linear regression was performed using the Ordinary Least Square linear regression model. Estimates having a standard error higher than 5% of the estimated value were excluded (Chemidlin Prévost-Bouré et al., 2009).

The same system was used to measure $\delta^{13}\text{C}_{F_i}$ and $\delta^{13}\text{C}_{F_m}$ on air samples taken from the laboratory incubation assays. A standard curve was established from the CO_2 concentration of standard air samples and the corresponding peak height, and used to determine the CO_2 concentration in the samples.

2.8. Statistical analysis

For each field session, measured variables were averaged per treatment as arithmetical means, excepted $\delta^{13}\text{C}_{F_S}$. Mean $\delta^{13}\text{C}_{F_S}$ ($\overline{\delta^{13}\text{C}_{F_S}}$) was calculated by weighting each estimate by its standard

error (Murtaugh, 2007). Equations are:

$$\overline{\delta^{13}\text{C}_{F_S}} = \sum_1^n w_i \times \delta^{13}\text{C}_{F_{S,i}} \quad (5)$$

with

$$w_i = \left(\frac{1/\text{SE}(\delta^{13}\text{C}_{F_{S,i}})^2}{\sum_1^n 1/\text{SE}(\delta^{13}\text{C}_{F_{S,i}})^2} \right) \quad (6)$$

$\text{SE}(\delta^{13}\text{C}_{F_{S,i}})$ is the standard error of the i th estimation of $\delta^{13}\text{C}_{F_S}$, and n the total number of observations.

The standard error of the mean was calculated as follows:

$$\text{SE}(\overline{\delta^{13}\text{C}_{F_S}}) = \sqrt{\left(\frac{1}{n-1} \right) \times \sum_1^n (w_i \times (\delta^{13}\text{C}_{F_{S,i}} - \overline{\delta^{13}\text{C}_{F_S}})^2)} \quad (7)$$

Table 1

Environmental conditions (soil water content, soil temperature at 10 cm depth); carbon content and isotopic composition of total soil organic carbon and “old” litter (naturally deposited during autumn 2005); total soil and bulk soil respirations (F_S and F_m , respectively) and their isotopic composition ($\delta^{13}\text{C}_{F_S}$ and $\delta^{13}\text{C}_{F_m}$, respectively) before treatment application. The isotopic composition of the litter used for the treatments ($\delta^{13}\text{C}$ of added litter) is also given. SE, standard error of the mean.

	E		C		A	
	Mean (\pm SE)	n	Mean (\pm SE)	n	Mean (\pm SE)	n
Soil water content (SWC, %)	20.8 (\pm 1.0)	4	20.4 (\pm 0.6)	4	21.9 (\pm 1.7)	4
Soil temperature ($T_{10\text{cm}}$, °C)	2.7 (\pm 0.5)	4	2.7 (\pm 0.5)	4	2.7 (\pm 0.5)	4
Soil organic carbon (% 0–10 cm)	1.71	1	2.63	1	1.47	1
Old litter (g m^{-2})	505 (\pm 73)	4	477 (\pm 73)	4	531 (\pm 107)	4
$\delta^{13}\text{C}$ of soil organic carbon (0–10 cm depth, ‰)	−26.19	1	−26.11	1	−26.06	1
$\delta^{13}\text{C}$ of old litter (‰)	−27.1 (\pm 0.1)	4	−26.9 (\pm 0.3)	4	−27.2 (\pm 0.2)	4
$\delta^{13}\text{C}$ of added litter (‰)			−27.95 (0.2)	16		
F_S ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	0.28 (\pm 0.03)	4	0.29 (\pm 0.03)	4	0.31 (\pm 0.04)	4
F_m ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	2.7 (\pm 0.9)	3	3.7 (\pm 0.4)	4	4.4 (\pm 0.3)	4
$\delta^{13}\text{C}_{F_S}$ (‰ versus PDB)	−24.4 (\pm 0.3)	3	−24.2 (\pm 0.4)	3	−24.1 (\pm 0.4)	2
$\delta^{13}\text{C}_{F_m}$ (‰ versus PDB)	−24.5 (\pm 0.7)	4	−24.3 (\pm 0.2)	4	−24.5 (\pm 0.4)	4

Non parametric paired tests (Friedman repeated measures one-way analysis of variance on ranks, cited as RMANOVA; and Wilcoxon's test for paired samples on ranks) were used to check for differences between the three treatments. Significance level was set at 5%. All statistical analyses were performed using Statistica software (Statsoft Inc., Tulsa, USA).

3. Results

3.1. Homogeneity of treatment plots before litter manipulation

The results of assessments carried out on the plots prior to the start of the experiment are reported in Table 1. No significant differences were observed for soil water content or soil temperature. Soil organic C content and its isotopic composition were similar in each treatment, as were "old" litter mass and isotopic composition. The treatments were also homogeneous in terms of F_S , F_m and their isotopic composition.

Additionally, the isotopic composition of soil organic C was significantly different from that of the fresh litter used for the experiment ($P < 0.05$, $\Delta_{\text{fresh litter-soil}} = -1.8\text{‰}$).

3.2. Soil temperature and moisture variations

Soil temperature ($T_{10\text{cm}}$) ranged between 2.7 and 18.5 °C with an annual mean of 11.4 °C (Fig. 1). The highest values were observed in summer. In March 2007, $T_{10\text{cm}}$ was 4.5 °C higher than values observed at the same period 1 year earlier because winter 2007 was particularly warm (Piao et al., 2008). Soil water content (SWC, 0–10 cm) was not affected by the quantity of fresh litter and ranged from 7.6% to 26.0% during the study period in the E, C and A plots. SWC variations were negatively correlated to $T_{10\text{cm}}$ variations ($|r| > 0.85$). SWC was lower than the "theoretical" wilting point threshold (estimated using the model of Saxton et al. (1986)) in July 2006, indicating a drought-constraint. This corresponds to a 15-day period (27 June–12 July 2006) during which only 10 mm of rain was recorded.

3.3. Fresh litter decomposition

In both C and A plots, an exponential decay function well fitted the data for litter dry mass loss with time ($r^2 > 0.7$). Litter degradation rate was not significantly different for the C and A treatments as the decay constants (k in Eq. (1)) in these treatments were not different: 2.01×10^{-3} (standard error: 6.47×10^{-5}) and 1.93×10^{-3} (standard error: 5.84×10^{-5}), respectively.

3.4. Response of total soil CO₂ efflux to variations in fresh litter amount

Fresh litter amount had a significant impact on F_S measured in the field (RMANOVA, $\chi^2 = 22$; $P < 0.0001$), as presented in Fig. 2. F_S was significantly reduced in the E treatment (by 25–45%) compared to the control (C, Wilcoxon's test, $P < 0.001$) while F_S was significantly increased in the A treatment (by 60–120% depending on the period considered) compared to the control (Wilcoxon's test, $P < 0.004$). No block effect was detected (Kruskal–Wallis test, $P > 0.5$). However, fresh litter addition or exclusion did not modify the seasonal trends of F_S . The maximum values occurred in June 2006 (2.2, 3.0 and $5.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ for E, C and A treatments, respectively) and the minimum in March 2007 (0.4, 0.5 and $0.9 \mu\text{mol m}^{-2} \text{s}^{-1}$ for E, C and A treatments, respectively). In July 2006, F_S was slightly reduced compared to June 2006 despite a higher soil temperature. In each treatment, the seasonal variations in F_S were well explained by a Q_{10} relationship to temperature ($r^2 > 0.8$). In this relationship, Q_{10} values (3.5, 3.2, 3.6 in the E, C

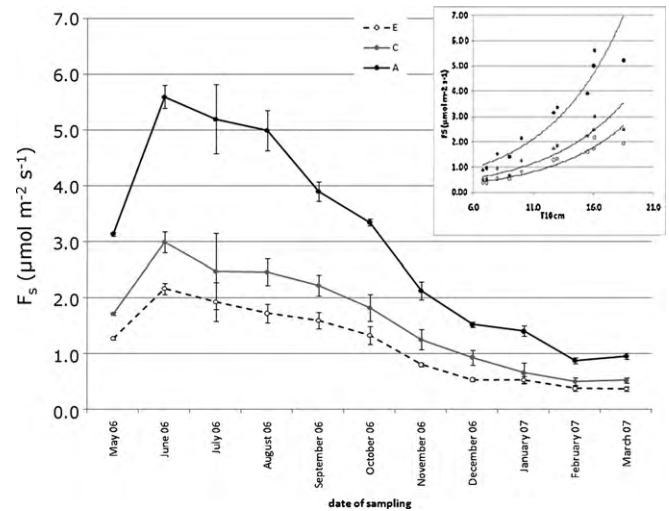


Fig. 2. Temporal variation in total soil CO₂ efflux (F_S) in the litter exclusion (E, dotted line), control (C, grey) and litter addition (A, black) treatments, respectively. Error bars correspond to one standard error of the mean. Inset graphic: response of F_S to soil temperature at 10 cm depth ($T_{10\text{cm}}$) in the litter exclusion (E, black filled circles), control (C, grey filled circles) and litter addition (A, open circles) treatments, respectively. A Q_{10} model: $F_S = R_{S10} \times Q_{10}((T_{10\text{cm}} - 10)/10)$; well fitted F_S temperature response. Q_{10} estimates: 3.5, 3.2, 3.6 in the E, C and A treatment, respectively. R_{S10} estimates: 0.8, 1.1, $2.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the E, C and A treatments, respectively.

and A treatments, respectively) were not affected by the treatment, whereas basal respiration at 10 °C (0.8, 1.1, $2.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the E, C and A treatments, respectively) was significantly ($P < 0.05$) changed by the fresh litter amount.

3.5. Role of litter in the modification of total soil CO₂ efflux intensity

To determine whether the significant variation in total soil CO₂ efflux intensity (F_S) could be attributed to the addition or the exclusion of fresh litter, the field measurements of F_S in the E and A treatments were compared to the expected total soil CO₂ efflux intensity (F_S^{EXPECTED}) in these treatments. In the A treatment, field measurements of F_S ($F_{S,A}$) were significantly higher ($P < 0.001$) than $F_{S,A}^{\text{EXPECTED}}$ (Fig. 3). Under these conditions, soil priming corre-

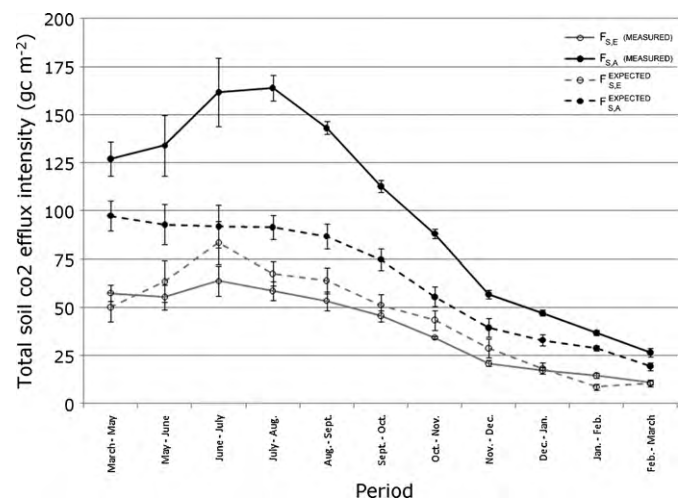


Fig. 3. Measured (*in situ*) and expected total soil CO₂ efflux over time in the litter exclusion (E) and litter addition (A) treatments across the experiment. Solid lines: measured total soil CO₂ efflux in the E (grey) and A (black) treatments. Dashed lines: expected total soil CO₂ efflux in the E (grey) and A (black) treatments. Error bars are standard errors of the mean ($n = 4$).

sponded to +32% of F_S measured in the A treatment. In the E treatment, F_S measured in the field ($F_{S,E}$) was not significantly different from $F_{S,E}^{EXPECTED}$ ($P > 0.2$), showing that litter decomposition in the control accounted for the difference in soil respiration between control and E treatment.

3.6. Response of heterotrophic CO₂ efflux in litter and soil to variations in fresh litter amount

Laboratory measurements of fresh litter CO₂ efflux (F_l) in the A treatment were twice as high as in the control (Fig. 4; Wilcoxon's test, $P < 0.01$), but differences were constrained by season (no differences in September 2006 and February 2007). Mean F_l was 0.13 and 0.22 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the C and A treatments, respectively. This difference was determined by fresh litter amount, since fresh litter respiration per g of dry mass was similar in the control and A treatments ($P < 0.60$). Fresh litter contribution to total soil CO₂ efflux ranged from 2% to 18% for both control and treatment A, and the higher litter contribution was observed during the period of leaf fall.

Heterotrophic CO₂ efflux in soil (F_m), measured in the laboratory, was not affected by litter addition (treatment A) or exclusion (treatment E) (Fig. 4b, RMANOVA, $\chi^2 = 4.9$, $P < 0.10$). Similar ranges of F_m were observed in all treatments: ca. 0.5–4 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The seasonal variations of F_m were also similar among the three litter treatments and were not related to incubation temperature.

3.7. The isotopic composition of total soil-respired CO₂ and heterotrophically respired CO₂ from litter and soil

Litter treatment did not significantly affect $\delta^{13}\text{C}_{F_S}$ measured in the field (RMANOVA, $\chi^2 = 3.8$, $P < 0.15$), which ranged from –27.9‰ to –22.5‰, in all treatments (Fig. 5a). Nevertheless, a trend was observed for $\delta^{13}\text{C}_{F_S}$ with CO₂ from treatment E being generally slightly ¹³C-enriched relative to other treatments.

Exclusion or addition of fresh litter did not affect $\delta^{13}\text{C}_{F_m}$ ($\chi^2 = 4.9$, $P < 0.09$, Fig. 5b). Ranges of variation of $\delta^{13}\text{C}_{F_m}$ were between –21.7‰ and –28.7‰ through the measurement period. $\delta^{13}\text{C}_{F_m}$ exhibited a clear increase in summer and a decrease during winter. CO₂ derived from heterotrophic respiration in soil was always ¹³C-enriched relative to total soil organic matter (mean apparent discrimination: 2.2‰).

Like $\delta^{13}\text{C}_{F_m}$, $\delta^{13}\text{C}_{F_l}$ was not affected by fresh litter addition ($P < 0.60$). Mean annual $\delta^{13}\text{C}_{F_l}$ was –25.9‰ and –26.3‰ in the control and A treatments, respectively (Fig. 5c). $\delta^{13}\text{C}_{F_l}$ was not different from $\delta^{13}\text{C}_{F_S}$ and most of the time was ¹³C-enriched relative to litter organic matter (mean apparent discrimination: 1.3‰ estimated

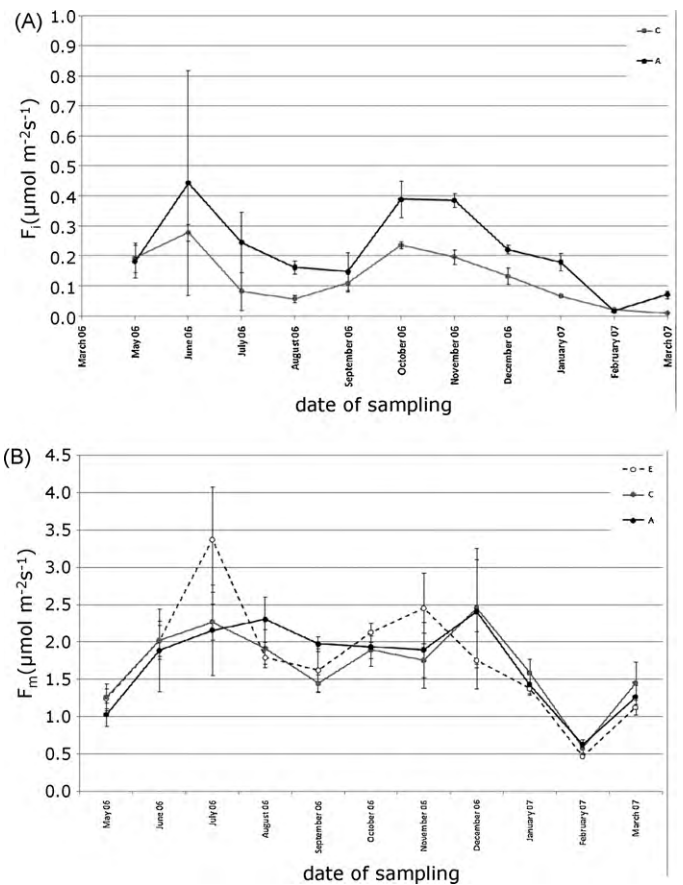


Fig. 4. Temporal variation in heterotrophic CO₂ efflux (A) in litter (F_l) in the control (C, grey filled circles) and litter addition (A, black filled circles) treatments; (B) in soil (F_m) in the litter exclusion (E, open circles), control (C, grey filled circles) and litter addition (A, black filled circles) treatments. Error bars represent the standard error of the mean ($n = 2-4$).

per date of sampling, data not shown). The range and the temporal dynamics of $\delta^{13}\text{C}_{F_l}$ were similar to those of the isotopic composition of root respired CO₂ (root excision method, data not shown).

4. Discussion

During the experiment, soil temperature and soil water content (SWC) were not modified by the treatment. A similar observation had been previously reported for soil temperature (Sulzman

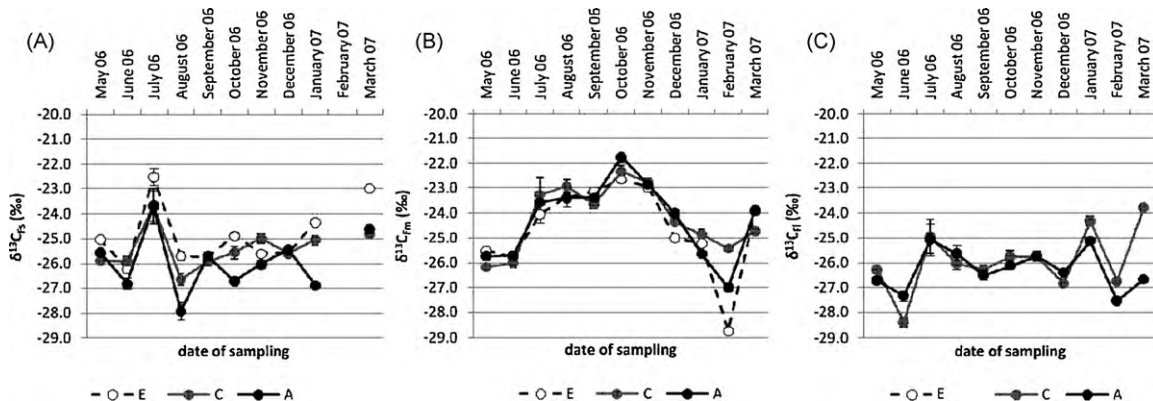


Fig. 5. Monthly variation in isotopic composition of respired CO₂ from total soil (A, $\delta^{13}\text{C}_{F_S}$), soil without roots (B, $\delta^{13}\text{C}_{F_m}$) and litter (C, $\delta^{13}\text{C}_{F_l}$) in the different litter treatments: litter exclusion (E, open circles), control (C, grey filled circles) and litter addition (A, black filled circles). $\delta^{13}\text{C}$ values are expressed in ‰ relative to the PDB standard. Error bars represent standard errors of the means calculated according to Murtaugh (2007). Number of observations for mean calculations ranged from 2 to 4.

et al., 2005) but not for SWC. This lack of variations of SWC may be explained by the dense canopy above the experimental area (leaf area index = 5.1; Delpierre et al., 2009) which strongly limited water evaporation from soil, particularly during the vegetative period (Rey et al., 2002). During this period, SWC variations may have mainly been driven by root water uptake. Added to that, the humus layer remained thin despite litter addition. Therefore, litter addition at our site may not have significantly modified the resistance of the humus layer to water infiltration into soil and water evaporation from soil (Baldocchi et al., 2000; Schäfer et al., 2002) throughout the experiment.

Temporal variations in F_S reported in this study for the different treatments (E, C and A) were in the range of those reported in the literature for different types of temperate deciduous forests: 0.5–6.0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Davidson et al., 1998; Hibbard et al., 2005; Vincent et al., 2006). The reduction in F_S observed in July 2006, while soil temperature was rising, was attributed to a drought event appearing in every treatment. In accordance with different field studies (Boone et al., 1998; Epron et al., 2004; Sulzman et al., 2005), the addition/exclusion of fresh litter significantly increased/decreased F_S , without modifying the contribution of fresh litter to F_S . These variations in F_S between treatment E, control and treatment A were not explained by variations in the temperature sensitivity of F_S (Q_{10}). Indeed, Q_{10} was not significantly modified by the quantity of fresh litter and differences in F_S between treatments were mainly explained by changes in basal soil respiration at 10 °C (F_{S10}). In every treatment, the Q_{10} value (3.2–3.6) was in the range of those reported in the literature (Boone et al., 1998; Davidson et al., 1998; Perrin et al., 2004; Gaumont-Guay et al., 2006), and was in the range of those corresponding to recalcitrant material according to Davidson and Janssens (2006) and Davidson et al. (2006). Therefore, the stability of the Q_{10} may be related to a higher consumption of soil organic matter relative to litter organic matter, which would limit the imprint of litter quantity itself on the Q_{10} . This hypothesis is supported by the relatively small contribution of F_1 to F_S (8% on average). Consequently, the variations in F_S and F_{S10} between treatments would be related to variations in soil activity mediated by the quantity of fresh litter.

Modifications of soil activity, and then of F_S and F_{S10} , may be related to changes in microbial biomass, according to the trend observed by Soe and Buchmann (2005), and/or to changes in soil microbial community composition (Nottingham et al., 2009), and/or changes in substrate availability or composition since microbial biomass is carbon-limited in soils (Kuzyakov et al., 2000; Ekblad and Nordgren, 2002; Fontaine et al., 2003; Crow et al., 2009). In our study, since no variation in soil microbial biomass was observed among treatments (Chemidlin Prévost-Bouré et al., in press), the hypothesis linking variation in F_S and F_{S10} between treatments to variation in microbial biomass is not supported. Significant seasonal variation in the genetic structure of soil and litter bacterial communities was observed during the experiment and also following treatment application. However, in contrast to litter, the variation in the genetic structure of the soil bacterial community was not statistically related to variation in F_S or F_m . Therefore, it is more likely that, in our case, the variation in F_S and F_{S10} between treatments was related to changes in substrate availability or composition in soil. This hypothesis is supported by field measurements: the reduction in F_S in the E treatment following litter exclusion was equivalent to litter respiration, and soil priming was detected in the A treatment as a consequence of the addition of fresh litter. But this hypothesis does not seem to agree with the laboratory measurements of F_m , which were not modified by litter addition or exclusion. Nevertheless, Crow et al. (2009) provided valuable evidence that soil priming may occur with no differences in F_m (shortly after the start of incubation) or even reduced F_m (at the end of the incubation) in the added litter plots relative to the

control plots. Added to that, it appears that very small differences in the determination of F_m (in a range close to that of the confidence interval of the measure) are likely to produce large differences in respiration rate when extrapolated to the soil column. In our study, such small differences may have been masked by the impact of soil sieving on CO_2 emission (Kuzyakov, 2006). Therefore, we may conclude that the variations in F_S and F_{S10} may have been determined by modification of substrate availability or composition in soil, promoting the use of soil C.

In accordance with the definition provided by Bingeman et al. (1953) and refined by Kuzyakov et al. (2000), soil priming detected in the A treatment corresponds to a “real” priming effect, i.e. a priming effect for which the carbon source corresponds to organic matter and not microbial biomass, which remained unchanged by the treatment (Blagodatskaya and Kuzyakov, 2008). The contribution of the priming effect estimated here (32% of F_S measured in the A treatment on average) was in the range of those reported *in situ* by Sulzman et al. (2005) and Crow et al. (2009). According to Fontaine et al. (2003) and Kuzyakov et al. (2000), the addition of fresh organic matter may have allowed the co-metabolic decomposition of more recalcitrant soil organic matter (SOM). This would be supported by Q_{10} estimates but not by the lack of variation in $\delta^{13}\text{C}_{F_S}$. Indeed, according to the linear mixing model (Phillips and Gregg, 2001), if only SOM had been mineralized during priming, the contribution of F_m to F_S would have been modified and then $\delta^{13}\text{C}_{F_S}$ would have been less negative in the A treatment than in the control, especially during the vegetative period when differences between $\delta^{13}\text{C}_{F_S}$, $\delta^{13}\text{C}_{F_1}$ and $\delta^{13}\text{C}_{F_m}$ were maximal (2–3‰), above the limit needed to discriminate between CO_2 sources (Phillips and Gregg, 2001). Because this increase was not observed, we may assume that primed respired CO_2 was not only derived from SOM but also from the rhizosphere as proposed by Subke et al. (2004) and that litter addition activated the whole soil system, accelerating C turnover processes. Further labelling experiments involving high-frequency carbon stable isotope measurements of CO_2 efflux would help to clarify the relative importance of bulk soil and rhizosphere in the priming effect.

Priming effect phenomena have been previously reported in laboratory studies (Hamer and Marschner, 2005; Kuzyakov and Bol, 2006; Fontaine et al., 2007), but only in a few field studies using complex carbon sources like litter (Subke et al., 2004; Sulzman et al., 2005; Crow et al., 2009). Here, F_S data suggest that the priming effect lasted for over 1 year, compared to a few days to weeks in laboratory studies. This long-lasting priming effect may be related to a continuous release of organic compounds from litter to soil because of the progressive decomposition of leaf litter. This is in agreement with the trends observed in the literature for the duration of a priming effect with increasing substrate complexity: e.g. Hamer and Marschner (2005) reported fast 1-day priming after addition of simple organic compounds (fructose, alanine), whereas Fontaine et al. (2007) and Nottingham et al. (2009) reported a priming effect that lasted 30 days after pure cellulose addition or chopped/ground maize litter, respectively.

Temporal variation in isotopic data can also be informative. The isotopic values were in the range of those reported in the literature for $\delta^{13}\text{C}_{F_S}$ (e.g. Ekblad and Hogberg, 2001; Bhupinderpal-Singh et al., 2003; Ngao et al., 2005) and for $\delta^{13}\text{C}_{F_m}$ (Andrews et al., 1999, 2000; Tu and Dawson, 2005). Both $\delta^{13}\text{C}_{F_S}$, $\delta^{13}\text{C}_{F_m}$ and $\delta^{13}\text{C}_{F_1}$ showed temporal variations throughout the experiment (amplitude of variations: from 2.4‰ to 6‰). Over the whole season, these temporal variations were not related to soil climatic conditions (soil temperature, SWC). Nevertheless, some of them have probably been influenced by climatic conditions: $\delta^{13}\text{C}_{F_S}$, $\delta^{13}\text{C}_{F_m}$ and $\delta^{13}\text{C}_{F_1}$ increased during the drought stress, which is in agreement with the response of photosynthesis to a drought stress (Farquhar et al., 1989) and the link between plant physiology and soil respiration (Högberg

et al., 2001). Plant physiology may not be the only determinant of $\delta^{13}\text{C}_{F_3}$, $\delta^{13}\text{C}_{F_m}$ or $\delta^{13}\text{C}_{F_1}$ temporal variations. Indeed, these variations could also be the result of changes in the isotopic composition of the mineralized organic compounds or in isotopic discrimination during organic matter decomposition (Santruckova et al., 2000; Wynn et al., 2006). These changes may be determined by variations in the composition of the soil microbial community that were demonstrated, in our study, to occur with time both in soil and litter (Chemidlin Prévost-Bouré et al., in press). Temporal variation in $\delta^{13}\text{C}_{F_1}$ was found to be significantly related to temporal variation in the litter bacterial community in the A treatment, but not in the other treatments and not in soil. Considering concomitantly temporal variation in $\delta^{13}\text{C}$ and microbial community composition may provide valuable clues to better understand organic matter mineralization dynamics and to test mineralization models dealing with microbial community variation (Neill and Gignoux, 2006). This would need “high frequency” isotopic measurements (CO_2 and organic matter) and isotopic labelling techniques to distinguish between active and non-active microbial populations.

5. Conclusions

Litter manipulation showed that soil CO_2 efflux was stimulated when fresh litter was added and was reduced when fresh litter was excluded. The observed activation was not determined by differences in soil climatic conditions (temperature and moisture) between treatments or by variation in temperature sensitivity (Q_{10}) of F_3 . Indeed, it appears to be the result of a real priming effect, for which the intensity may be affected by seasonal variation in soil climatic conditions. This priming effect represented a large proportion of soil CO_2 efflux in the litter addition treatment and probably resulted in the activation of the whole soil system. This study highlights the complexity of direct and indirect interconnections between soil carbon source components in the context of climate change as modifying the size of the young carbon pools (here litter) may lead to the fast remobilization of older soil C stocks through respiration, particularly in temperate forests. In addition, this study highlights the importance of labelling experiments to improve our knowledge of litter–soil interaction and to decipher the mechanisms of organic matter mineralization.

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Biogeographical patterns of soil bacterial communities

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Summary

This study provides the first maps of variations in bacterial community structure on a broad scale based on genotyping of DNA extracts from 593 soils from four different regions of France (North, Brittany, South-East and Landes). Soils were obtained from the soil library of RMQS ('Réseau de Mesures de la Qualité des Sols' = French soil quality monitoring network). The relevance of a biogeographic approach for studying bacterial communities was demonstrated by the great variability in community structure and specific geographical patterns within and between the four regions. The data indicated that the distribution of bacterial community composition might be more related to local factors such as soil type and land cover than to more global factors such as climatic and geomorphologic characteristics. Furthermore, the regional pools of biodiversity could be ordered: South-East \geq North > Brittany > Landes, according to the observed regional variability of the bacterial communities, which could be helpful for improving land use in accordance with soil biodiversity management.

Although microorganisms are the most ubiquitous, diverse and abundant living organisms on earth and despite their key role in biogeochemical cycles, in comparison with macroorganisms, few investigations have been carried out on the distribution of soil microbial community diversity on a broader scale than agricultural fields or forest sites. Indeed, most studies have focused on cataloguing the microbial diversity in particular sites and describing how communities have been affected by environmental perturbations (for review see Ranjard *et al.*, 2000) without integrating the spatial scale in microbial community assembly (Papke and Ward, 2004). As a result, the survey of microbial diversification and the distribution patterns of microbial diversity on a large scale are poorly documented and understood (Martiny *et al.*, 2006). Ecologists have long recognized that beta-diversity (how community composition changes across a landscape) can offer valuable insights into the relative influence of dispersal limitations, environmental heterogeneity and environmental and evolutionary changes in shaping the structure of ecological communities (Green *et al.*, 2004).

In this context, the aim of our study was to investigate the geographic distribution patterns of bacterial community structure by considering a broad spatial scale of sampling. For this, we characterized the bacterial communities from soils in the RMQS soil library ('Réseau de Mesures de la Qualité des Sols' = French soil quality monitoring network) (Fig. 1). This library represents 2200 soils sampled with a 16 km \times 16 km systematic grid over the entire French territory and representative of the different land uses, soil types and climatic conditions occurring in France (for more details see Arrouays *et al.*, 2002 and Supporting information). We focused on characterizing the bacterial community structure in 593 soils sampled from four different geographical regions (North, Brittany, Landes and South-East, Fig. 1). These regions were chosen for their particular geographic, pedo-climatic and land use characteristics (as described in the JRC Soil Atlas of Europe, http://eusoils.jrc.ec.europa.eu/projects/soil_atlas). The soil bacterial community structure was directly genotyped from soil DNA extracts, using a B-ARISA (Bacterial-Automated Ribosomal Intergenic Spacer Analysis) fingerprinting approach (Ranjard *et al.*, 2003) optimized for medium throughput in the platform GenoSol (http://www.dijon.inra.fr/plateforme_genosol). The ARISA fingerprinting was used as it allows the rapid examination of the genetic structure of complex bacterial

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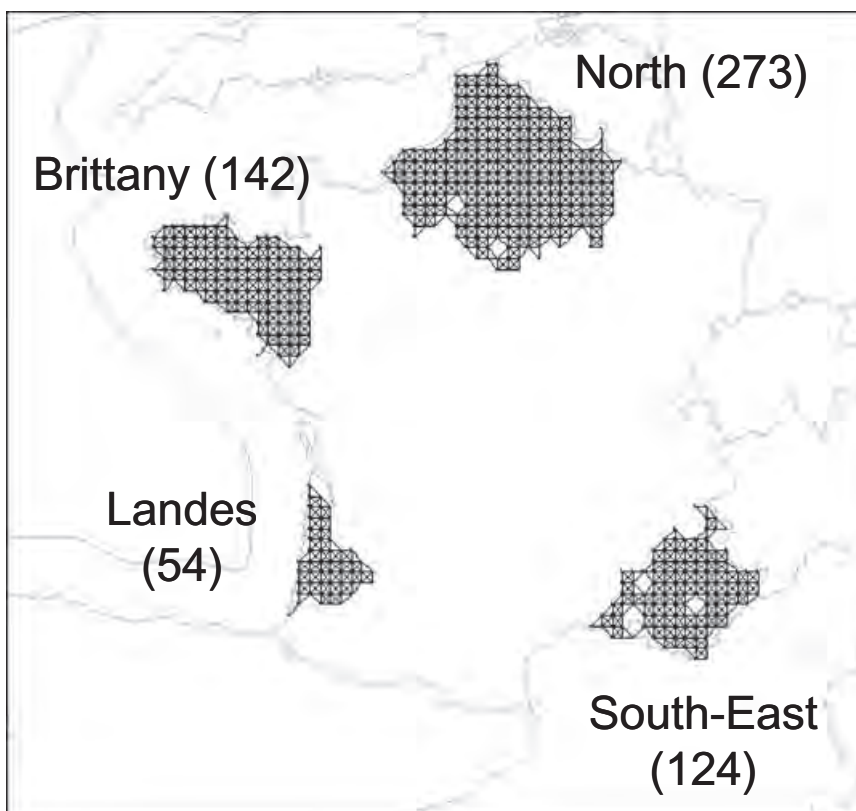


Fig. 1. Location and delimitation of the four studied regions in France. Numbers in parentheses indicate the number of soils samples analysed in each region. The neighbouring relationship between the sampling sites is indicated on the map (this relationship corresponds to a one-square move of the Queen on a chess board: each site can have up to eight neighbours).

communities and has been demonstrated to be sensitive and relevant for evaluating modifications in community composition in space and time (Ranjard *et al.*, 2001; 2003).

Complex B-ARISA profiles were obtained for each soil and compiled into a single matrix (Ranjard *et al.*, 2003), which was analysed using a generalization of multivariate spatial correlation analysis called MULTISPATI (see Dray *et al.*, 2008 and Supporting information for a detailed description of this method). This analysis takes into account the spatial position of sampling sites on the basis of neighbouring relationships between sites (see Fig. 1). One of the biggest advantages of MULTISPATI scores is that they maximize spatial autocorrelation. Consequently, they can be used to draw easily interpretable geographical maps. Computations were conducted using the R software (R Development Core Team, 2008), with the *ade4* (Chessel *et al.*, 2004; Dray and Dufour, 2007) and *spdep* (Bivand *et al.*, 2008) packages (for more details see the 'Materials and Methods' section in the Supporting information file).

The factorial map deduced from the MULTISPATI analysis of all B-ARISA profiles demonstrated the considerable variability of the bacterial community structure

within and between the four different regions (for more detailed results see Supporting information for Fig. S1). Significant regional and national variations of soil bacterial communities were apparent, which were consistent to those observed on micro- or field scales (Ranjard and Richaume, 2001; Ranjard *et al.*, 2001) and with other studies on broader spatial scales (Fierer and Jackson, 2006). The site scores of the first three axes of global MULTISPATI analysis were first interpolated using a geostatistical interpolation method and then geographically mapped for each studied region (Fig. 2) to determine the distribution patterns of soil bacterial community variability (for more details see Supporting information). Mapping of the first MULTISPATI score revealed a moderate regional variability within Landes and Brittany and a high similarity in community structure between these two regions (Fig. 2 Axis 1). In contrast, the North and South-East regions exhibited much greater regional variability and distinct community structures compared with Landes and Brittany. Mapping of the second MULTISPATI score (Fig. 2 Axis 2) discriminated between Brittany and Landes as well as between North and South-East whereas mapping of the third MULTISPATI score (Fig. 2 Axis 3) revealed greater regional heterogeneity and the evidence of new spatial

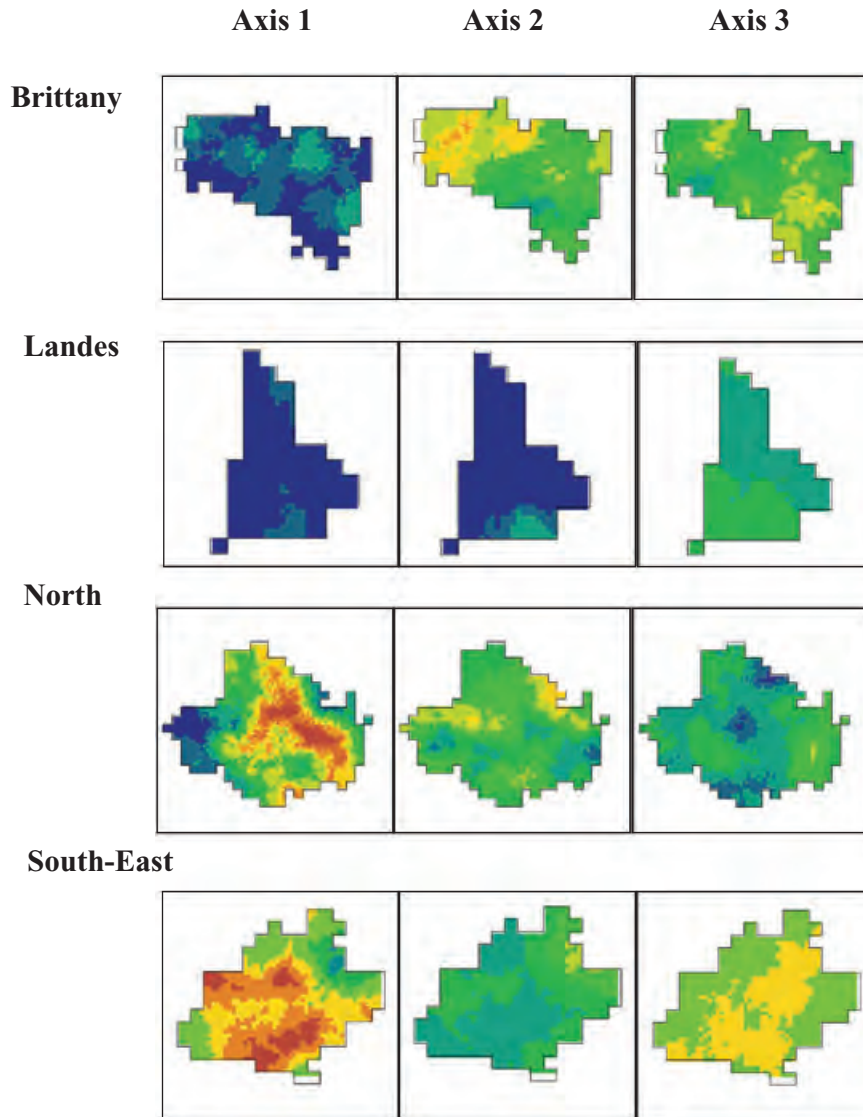


Fig. 2. Maps of interpolated MULTISPATI scores for the first three MULTISPATI axes (columns) and for the four geographical regions (rows). Each map is a spatial synthesis of the B-ARISA genetic structure of indigenous bacterial communities from the corresponding soils sampled in the four regions of France.

Axis 1, Axis 2 and Axis 3 represent the mapping of MULTISPATI scores for the first three axes. Colours on the map are proportional to the score values (see Fig. S1 in Supporting information), and the colour code is given by the following scale:



Similar colours and scores on a given axis indicate similar genetic structure of bacterial community.

structures. On the basis of these maps, the null hypothesis of microbial biogeography, corresponding to a random spatial distribution of microorganisms, could be rejected (Martiny *et al.*, 2006). These maps further evidenced that bacterial community can have biogeography with significant and specific spatial assemblages according to the studied region. Interestingly, similar community structures appeared to occur in non-contiguous regions, suggesting that environmental conditions (geomorphologic, pedo-climatic, land cover ...), rather than geographic distance, could be of major importance in shaping community structure (Martiny *et al.*, 2006). This observation contrasts with previous suggestions that geographic distance could be a useful predictor of microbial community turnover and assemblage for other types of ecosystems (mountain lakes, Reche, 2005) as well as for other types of microorganisms (fungi in desert ecosystem, Green *et al.*, 2004).

The particular and homogeneous assemblage of bacterial communities in the Landes and Brittany regions could not be explained by a geographic isolation of these regions due to the presence of natural barriers (mountain, sea, desert . . . ; http://eusoils.jrc.ec.europa.eu/projects/soil_atlas, elevation p. 121) or by particular climatic conditions (http://eusoils.jrc.ec.europa.eu/projects/soil_atlas, climate p. 122). However, the mapping of French soil types according to their physico-chemical characteristics (<http://gissol.oreans.inra.fr/programme/bdgsf/carte.php>) appeared to match well with the bacterial community distribution within and between regions, supporting the hypothesis of a strong influence of edaphic parameters. More precisely, in the Landes region a single sandy acidic soil type (podzol) has been described for all the studied sites (IUSS Working Group WRB report 2006), which might partly explain the low variability of the bacterial community in this region. This hypothesis is supported by previous studies dealing with the description of bacterial diversity on continental or field scales that demonstrated the more important influence of soil characteristics, such as pH (Fierer and Jackson, 2006) and/or soil texture (Johnson *et al.*, 2003), than climatic or land cover characteristics. The higher variability of soil types described in the North and South-East regions (<http://gissol.oreans.inra.fr/programme/bdgsf/carte.php>) might support also a relationship between soil-type distribution and community assemblage on a broad scale. In parallel, Mantel tests and partial Mantel tests were used to investigate the relationships between geographical distances, physico-chemical characteristics of soil samples (texture, pH, CEC, Corg, N, CaCO₃, P, Ca, Mg, K contents) using Euclidean distances, and Sørensen similarity coefficient between B-ARISA profiles. All computations were done with the R software (R Development Core Team, 2008), using the vegan (Oksanen *et al.*, 2009) and labdsv

(Roberts, 2007) packages. A simple Mantel test showed that there is a very highly significant correlation between B-ARISA profiles and soil physico-chemical characteristics ($r = 0.326$, $P < 0.001$). There is also a significant correlation between B-ARISA profiles and geographical distances ($r = 0.225$, $P < 0.001$). But, after controlling for geographical distances, a partial Mantel test showed that there is still a very highly significant correlation between B-ARISA profiles and soil physico-chemical characteristics ($r = 0.277$, $P < 0.001$).

The spatial variation of community structure might also be related to the more or less regional variability of land cover (<http://image2000.jrc.it/>; http://eusoils.jrc.ec.europa.eu/projects/soil_atlas, land cover p. 123). The Landes region is mainly characterized by a monospecific forest cover with (monoclonal) *Pinus pinaster*, intensively exploited for wood production. Such a homogenous over exploitation of soil in this region might be consistent with the low regional variability observed for community assemblage. In contrast, the higher variability recorded in the three other regions could be related to the greater land use variability, which includes forest, grassland and agricultural crops. Consequently, the Landes region can be defined as a biotic province with very few types of particular habitats in terms of climatic, pedological and land cover characteristics, leading to a homogenous distribution of bacterial community composition.

This study represents the first exploratory step of an extensive biogeographical study to be applied to the whole French territory. Although our results remain descriptive, they implicitly support the second hypothesis of Bass-Becking (1934) deduced from the work of Beijerinck (1913), i.e. '*everything is everywhere*, but, *the environment selects*', implying that different contemporary environments maintain distinctive microbial assemblages. In other respects, our data also evidenced that microbial biogeography differs fundamentally from the biogeography of macro-organisms, which appears to be more influenced by global parameters such as climate or geomorphology (Green and Bohannan, 2006). Finally, regional pools of microbial diversity could be ordered according to the regional variability of the bacterial community structure, i.e. South-East \geq North > Brittany > Landes, thus demonstrating the need to better understand the biogeographical patterns of microbial communities in order to improve our capacity to manage and protect soil biological diversity on a large scale.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Factorial map obtained by MULTISPATI analysis of B-ARISA profiles of soils sampled in the four regions studied. (A) Axis1 × Axis 2; (B) Axis 1 × Axis 3.

Appendix S1. Materials and methods.

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Root exclusion through trenching does not affect the isotopic composition of soil CO₂ efflux

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Abstract Disentangling the autotrophic and heterotrophic components of soil CO₂ efflux is critical to understanding the role of soil system in terrestrial carbon (C) cycling. In this study, we combined a stable C-isotope natural abundance approach with the trenched plot method to determine if root exclusion significantly affected the isotopic composition ($\delta^{13}\text{C}$) of soil CO₂ efflux (R_S). This study was performed in

different forest ecosystems: a tropical rainforest and two temperate broadleaved forests, where trenched plots had previously been installed. At each site, R_S and its $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{R_S}$) tended to be lower in trenched plots than in control plots. Contrary to R_S , $\delta^{13}\text{C}_{R_S}$ differences were not significant. This observation is consistent with the small differences in $\delta^{13}\text{C}$ measured on organic matter from root, litter and soil. The

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lack of an effect on $\delta^{13}\text{C}_{\text{R}_\text{S}}$ by root exclusion could be from the small difference in $\delta^{13}\text{C}$ between autotrophic and heterotrophic soil respirations, but further investigations are needed because of potential artefacts associated with the root exclusion technique.

Keywords Stable carbon isotopes · Natural abundance · Soil respiration · Trenched plot · Rainforest · Temperate forest

Introduction

Soil plays an important role in the ecosystem carbon (C) cycle by sequestering as much as 70% of total forest ecosystem C as organic matter (Malhi et al. 1999) and contributing 40–70% to total annual forest respiration through soil respiration (R_S) (Chambers et al. 2004; Epron et al. 2004a; Janssens et al. 2001; Bonal et al. 2008). As a consequence, R_S is a major component determining the source or sink status of a forest ecosystem.

Several studies have focused on R_S in various ecosystem types and all reported it was highly spatially and temporally variable (Buchmann 2000; Epron et al. 2004a, b; Fang and Moncrieff 2001; Gaumont-Guay et al. 2006; Högberg et al. 2001; Longdoz et al. 2000; Saiz et al. 2006; Salimon et al. 2004; Yim et al. 2003). Both spatial and temporal variability of R_S were related to variations in environmental factors and in C inputs via roots or litter (Epron et al. 2004b; Fang et al. 1998; Högberg et al. 2001; Longdoz et al. 2000) affecting the use of two C pools in soil (Epron et al. 2001): (1) a short residence-time C pool (fast C pool) of photosynthetic assimilates and root exudates that are respired as autotrophic respiration (R_a) by roots and associated microorganisms; and (2) a long residence-time C pool (slow C pool) of litter and soil organic matter (SOM) that are respired as heterotrophic respiration (R_h) mainly by microorganisms not associated with roots. Disentangling the processes using these two C pools is decisive in understanding soil functioning and modeling soil respiration in terrestrial ecosystems in the context of global environmental change (Baggs 2006).

To separate the components of soil respiration that uses either the slow or fast C pools, different methods are applied. Non-isotopic methods are based on cutting off the fast C pool inputs either by trenching (Boone et al. 1998; Buchmann 2000; Lee et al. 2003; Li et al.

2006) or tree girdling (Gottlicher et al. 2006; Högberg et al. 2001). Applying these methods on different ecosystems significantly reduces R_S and allows calculation of the heterotrophic contribution to R_S (see the review of Subke et al. 2006). However, these methods are still subject to uncertainties linked to decomposition of severed roots or soil water content differences among plot types (Ngao et al. 2007).

C isotope-based methods are extensively used to study soil functioning. There have been many *in situ* measurements of the isotopic composition of soil-respired CO_2 in both C_3 and C_4 ecosystems (Buchmann et al. 1997; Davidson 1995; Ekblad et al. 2005; Fessenden and Ehleringer 2003; Steinmann et al. 2004). These methods allow targeting of specific soil processes via isotopic labeling (Andrews et al. 1999; Kuzyakov et al. 2001; Ngao et al. 2005) or picturing the whole soil functioning at natural abundance. This last method successfully separated the R_a and R_h components of R_S in ecosystems characterized by C_3 / C_4 successions (Cheng 1996; Rochette et al. 1999). This separation was possible because the isotopic compositions (through the $\delta^{13}\text{C}$ notation) of the fast and slow C pools were very different ($> 10\text{‰}$). However, most terrestrial ecosystems do not have such large differences between the fast and slow C pools, *i.e.* differences in $\delta^{13}\text{C} < 2\text{‰}$ are common (Balesdent et al. 1993; Bowling et al. 2008). In a review on C_3 ecosystems, *in vitro* root respiration was clearly depleted compared to *in vitro* soil respiration (Bowling et al. 2008). On the contrary, microbially respired CO_2 seems to be ^{13}C enriched by 1–4‰ compared to bulk SOM (Andrews et al. 1999; Bowling et al. 2008; Tu and Dawson 2005). Such observations should be confirmed *in situ*, but similar discrepancies would be expected between autotrophic and heterotrophic soil respirations. According to Phillips and Gregg (2001), such discrepancies would lead to accurate estimates of autotrophic and heterotrophic contributions to total R_S . Therefore, it is valid to determine if natural abundance of stable C-isotopes can separate R_a and R_h in ecosystems with small differences of isotopic composition between the slow and fast C pools. To our knowledge, only two studies tackled this question using soil static-chamber methods. In a boreal forest, Subke et al. (2004) showed that CO_2 efflux from soil without root respiration (girdled plots) was ^{13}C -depleted compared to rooted soil (ungirdled plots), but differences were not

significant. In another study, Formanek and Ambus (2004) performed an *in vitro* / *in situ* experiment and showed that $\delta^{13}\text{C}$ of root respiration was in—between those of soil humus and mineral horizons respiration.

The main objective of this study was to determine whether $\delta^{13}\text{C}$ of soil CO_2 efflux measured *in situ* was significantly affected by root exclusion in different ecosystems. Keeling plots (Keeling 1958) made with closed dynamic systems in a tropical evergreen forest (Paracou, French Guyana) and in two temperate deciduous forests (Barbeau and Hesse, France) were performed to compare the isotopic composition of CO_2 evolved from control and trenched plots.

Material and methods

Experimental sites

The Paracou site (French Guiana, 35 m elevation) is located in the Paracou Research Station (Gourlet-Fleury et al. 2004) within the “Guyaflux experimental unit” that covers 200 ha of tropical wet forest (Bonal et al. 2008). Mean annual air temperature and rainfall over the past 10 years (1998–2008) were 25.7°C and 3,041 mm, respectively. Soils are mostly acrisols (FAO-ISRIC-ISSS 1998) developed over a Precambrian metamorphic formation called the “Bonidoro series” and composed of schist and sandstone.

The Hesse experimental sites (Hesse 1 and Hesse 2) are in the Hesse National Forest (CARBOEUROPE site, northeastern France, 300 m elevation; Granier et al. 2000). The Hesse 1 site is in the center of a 65 ha stand of 35 year-old European beech (*Fagus sylvatica* L.). The Hesse 2 site is in a mixed 20 year-old stand dominated by European beech. Mean annual air temperature and precipitation are 9.2°C and 820 mm, respectively. The soil is a stagnic luvisol (FAO-ISRIC-ISSS 1998) of 120 cm depth covered by an oligo-mull humus.

The Barbeau experimental sites (Barbeau 1 and Barbeau 2) are located in Barbeau National Forest (CARBOEUROPE site, southeast of Paris, France, 90 m elevation). The Barbeau 1 site is located in an oak (*Quercus petraea* L.) high forest stand. The Barbeau 2 site is located in an oak forest with coppiced hornbeam stand. Mean annual air temperature and rainfall are 10.7°C and 690 mm, respectively (1980–1996). Soil is a gleyic luvisol (FAO-ISRIC-ISSS

1998) of 80 cm depth, developed on millstone bedrock and covered by an oligo-mull humus.

All sites belong to the French network of forest ecosystems (Observatoire de Recherche en Environnement “fonctionnement des écosystèmes forestiers”).

Experimental design

Trenched plots (TP) were installed at each experimental site to suppress the autotrophic component of total soil respiration. Trenches were dug around areas without trees and the delimited plots were lined using a thick plastic film to prevent external root ingrowth. One control, untrenched plot (CP), was selected near each TP. TP and CP characteristics for each experimental site are summarized in Table 1. At Paracou site, trenches were dug down to a stone line composed of coarse fragments of lithorelics (60 cm depth). At Barbeau sites, they were dug down to the bedrock (80 cm depth). At Hesse sites, trenches were dug down to the clay horizon (90 cm depth). In all cases, these layers are almost impermeable to roots, preventing root ingrowth. At the time of the measurements, at any site, there was no evidence of root ingrowth into the TP plots. At Paracou sites, root ingrowth was observed only several months after the completion of this study, associated to a sharp increase of soil respiration in the TP plots (data not shown). At Barbeau and Hesse sites, such an increase of soil respiration was not observed (data not shown).

Measurements started at least 1 month after trenching (Lee et al. 2003), with details in Table 1. The measurements were carried out during the main phenological phases: leafy and unleafy seasons in temperate forests (Hesse and Barbeau); or during the main climatic periods: wet and dry periods in the tropical forest (Paracou). At Hesse sites, measurements were in March, May and September 2005. At Barbeau sites, measurements were from March 2005 to May 2006. At the Paracou site, measurements were at the end of the long dry season of August–November 2004, and during the short dry period of March 2005 that interrupts the rainy season of December–July.

Isotopic composition of soil CO_2 efflux

The isotopic composition of soil CO_2 efflux ($\delta^{13}\text{C}_{\text{RS}}$) was determined on CP and TP using the Keeling plot method (Keeling 1958). Soil CO_2 efflux was mea-

Table 1 Site characteristics and description of trenched (TP) and control (CP) plots. t refers to the time delay between trenching and measurements. nd: not determined

Site	Paracou	Barbeau 1	Barbeau 2	Hesse 1	Hesse 2
Location	5°16' N; 52°54' W	48°29' N; 02°47' E	48°29' N; 02°47' E	48°40' N; 7°05' E	48°40' N; 7°05' E
Climate	Tropical	Modified temperate maritime	Modified temperate maritime	Continental	Continental
Stand type	Mixed forest	Oak high forest	Oak forest with coppiced hornbeam	Beech high forest	Mixed beech forest
Stand age in 2006 (years)	nd	100 to 150	100 to 150	35	20
Ground area (m ha ⁻¹)	nd	20.7	20.7	25.3	25.3
Max tree height (m)	35–40	30	30	20	20
Dominant species	nd	<i>Quercus petraea</i> L.	<i>Quercus petraea</i> L.	<i>Fagus sylvatica</i> L. <i>Quercus petraea</i> L. <i>Betula pendula</i> L. <i>Carpinus betulus</i> L.	<i>Fagus sylvatica</i> L. <i>Quercus petraea</i> L. <i>Betula pendula</i> L. <i>Carpinus betulus</i> L.
Understorey	> 140 sp. ha ⁻¹	<i>Carpinus betulus</i> L.	<i>Carpinus betulus</i> L.	15	15
Represented species	6	1	1	1 / 1	1 / 1
t (month)	4 / 4	1 / 1	1 / 1	9 / 35	9 / 35
Number of TP / CP	0.64 / 0.64	6 / 6	6 / 6	0.9	0.9
Area of TP / CP (m ²)	0.6	0.8	0.8	3	3
Trenching depth (m)	2	10	5	2 to 4 / 2 to 4	2 to 4 / 2 to 4
Number of field sessions	4 / 4	10 / 1	10 / 1		
Measurements per field session (R _S / δ ¹³ C _{R_S})	4 / 4				

sured by an accumulation chamber connected to an infrared gas analyzer (EGM 1 or EGM4, PP Systems, Hitchin, UK, in Barbeau and Paracou, respectively; and LI-6200, Li-Cor Inc, Lincoln, NE, USA, in Hesse) in closed circuit mode. The Li-Cor chamber model Li-600-9 was used at Hesse, while laboratory-made chambers were used at Barbeau and Paracou sites. These two chambers were constituted of a Perspex (acrylic resin) cylinder (25.4 dm³, 12 cm height in Barbeau and 5 dm³, 52 cm height in Paracou) equipped with a small fan (airflow 13.5 m³ h⁻¹). During measurements, soil respiration chambers were laid on permanent collars inserted 2–3 cm into soil (one per TP and one per CP at Paracou and Barbeau; three per CP and two per TP at Hesse). Chambers were equipped to allow air sampling during measurements

Air samples were collected every 50–100 ppm CO₂ increase in the range 400–1,000 ppm. At Barbeau sites, five air samples were collected during CO₂ increase using 50 cm³ valved syringes (SGE, Australia) directly connected to the chamber. At both Hesse and Paracou sites, 5–6 air samples were collected during CO₂ increase using a specific sampling device allowing air to be driven from the chamber into 10 cm³ Exetainer glass vials (Labco, High Wycombe, UK), and closing of the Exetainer vial in airtight conditions. This system is described in detail by Ngao et al. (2005). At the time of syringe collection (Barbeau) or Exetainer cap closure (Hesse and Paracou), the CO₂ concentration was recorded. The time lag between detection in the gas analyzer and the vial collection was < 1 s due to the flow rate of the instruments (0.3–1.5 L min⁻¹). The expected [CO₂] changes during these time lags were within the range of instrumental error (1 ppm).

The isotopic composition of sampled CO₂ (δ¹³C_{CO2}) was determined using isotopic ratio mass spectrometers (IRMS): a VG Optima IRMS (Fison, Villeurbanne, France) connected to an elemental analyzer (model NA-1500, Carlo Erba, Milan, Italy) for the Barbeau samples, and a Delta S IRMS (Delta S, ThermoFinnigan, Bremen, Germany) connected to a gas purification device (Gas-Bench II, ThermoFinnigan) for the samples from Hesse and Paracou. The methods cited above are described in Maunoury et al. (2007) for the Barbeau samples; and in Ngao et al. (2005) for Hesse and Paracou. Analyses were performed within a week after each field session.

Isotopic analysis standard error was 0.2%. All isotopic composition values are expressed relative to the international PDB standard.

Isotopic composition of organic matter

Soil (0–15 cm) and aboveground litter were sampled during each field session at Paracou and Hesse. At Barbeau, soil and aboveground litter were sampled during three field sessions. For each sampling session, one measurement per CP plot was performed. Each measurement was performed on one soil sample at Paracou and on a composite sample (3 soil cores) at Hesse and Barbeau. Soil was sieved to 1 mm. All fine roots (< 1 mm diameter) were separated from bulk soil after sieving. Samples were dried at 60°C and ground. Ground litter, root and soil samples were analyzed for $\delta^{13}\text{C}$ determination using an elemental analyzer (model NA-1500) connected to the IRMS.

Soil respiration (R_S) measurements

At Hesse and Paracou sites, R_S was measured before air sample collection using the same chamber as previously described for the determination of $\delta^{13}\text{C}_{R_S}$. At the Barbeau sites, R_S was measured on 10 collars each in the CP and TP. These collars were inserted 2 cm into the soil in the vicinity of the Keeling plot collar. Measurements were performed with a soil respiration chamber (SRC-1, PP systems) linked to a CIRAS-1 gas analyzer (PP systems). Wind speed, measured inside the chamber with a thermal anemometer (Testo, Model Lenzkirch, Germany) was 0.4 m s^{-1} as recommended in Le Dantec et al. (1999). In addition, no system presented any difference in pressure between the headchamber and the outside, arguing for no major leak and no perturbation of the CO_2 diffusion gradient due to over/underpressure (Longdoz et al. 2000).

Data analysis

In each forest, R_S recorded on TP were not corrected for dead root decomposition, and arithmetic means of R_S were calculated for each field session: 10 collars at Barbeau, 4 at Paracou, and 2–4 at Hesse sites.

The $\delta^{13}\text{C}_{R_S}$ was estimated from a linear regression fitted through “transformed” CO_2 concentration ($[\text{CO}_2]$) and $\delta^{13}\text{C}_{\text{CO}_2}$ values. We tested two different transformation methods. Firstly, a linear regression was

fitted through the inverse of $[\text{CO}_2]$ (independent variable) and $\delta^{13}\text{C}_{\text{CO}_2}$ (dependent variable) where $\delta^{13}\text{C}_{R_S}$ was the intercept (Keeling 1958). Secondly, a linear regression was fitted through $[\text{CO}_2]$ (independent variable) and the product of $[\text{CO}_2]$ and $\delta^{13}\text{C}_{\text{CO}_2}$ (dependent variable) where $\delta^{13}\text{C}_{R_S}$ was the slope (Miller and Tans 2003). In both cases, we used both ordinary least squares linear regression (model I) and geometric mean linear regression (model II) (Sokal and Rohlf 1995). Model I assumes that the independent variable is measured without random error and the optimization of the regression parameters is made to minimize errors on the dependent variable. Model II is more robust since it assumes that both dependent and independent variables are measured with some error. Model II optimizes the regression parameters to minimize errors on both dependent and independent variables (Sokal and Rohlf 1995). The correlation coefficient (r) was calculated in each case.

The standard error of $\delta^{13}\text{C}_{R_S}$ was determined as the standard error of either the intercept or the slope of the regression when Keeling or Miller-Tans methods were used, respectively.

In Hesse and Paracou, mean $\delta^{13}\text{C}_{R_S}$ ($\overline{\delta^{13}\text{C}_{R_S}}$) values obtained over replicate collars and the standard error of the mean ($SE(\overline{\delta^{13}\text{C}_{R_S}})$) were calculated as described in Murtaugh (2007). In this method, mean $\delta^{13}\text{C}_{R_S}$ and its standard error are corrected for the error made on each estimation.

$$\overline{\delta^{13}\text{C}_{R_S}} = \sum_{i=1}^n w_i * \delta^{13}\text{C}_{R_S,i} \quad (1)$$

$$\text{with } w_i = \left(\frac{\frac{1}{SE(\delta^{13}\text{C}_{R_S,i})^2}}{\sum_{i=1}^n \frac{1}{SE(\delta^{13}\text{C}_{R_S,i})^2}} \right) \quad (2)$$

$SE(\delta^{13}\text{C}_{R_S,i})$ is the standard error of $\delta^{13}\text{C}_{R_S}$ i^{th} estimation, and n the total number of observations ($n=4$ in Paracou; $n=2-4$ in Hesse).

$$SE(\overline{\delta^{13}\text{C}_{R_S}}) = \sqrt{\left(\frac{1}{n}\right) * \sum_{i=1}^n \left[w_i * \left(\delta^{13}\text{C}_{R_S,i} - \overline{\delta^{13}\text{C}_{R_S}} \right)^2 \right]} \quad (3)$$

Overlapping of confidence intervals calculated from the standard error of the mean in Hesse and

Paracou, and from the standard error of the parameter in Barbeau (no replication), was used as a criterion to determine if $\delta^{13}\text{C}_{\text{R}_s}$ was significantly different between TP and CP for each date of measurement. The same criterion was used to test if $\delta^{13}\text{C}$ differed between root, soil and litter bulk organic matter. Treatment effect on R_s was tested by one way analysis of variance for repeated measurements (one way RM-ANOVA) on raw data. Significance level was $P < 0.05$.

Results

Comparison of $\delta^{13}\text{C}_{\text{R}_s}$ estimation methods

The different transformation methods and regression models were compared using the complete dataset from the three forests. First, Miller–Tans and Keeling transformations were compared using the same model (linear regression model I in Fig. 1a; model II in

Fig. 1b). $\delta^{13}\text{C}_{\text{R}_s}$ estimated by Keeling method was similar to that estimated by Miller–Tans method either when linear regression model I or model II were applied (slopes=0.98 and $r=0.98$). In each case, slopes were not significantly different from 1.

Model I and II linear regressions were compared for the same variable transformation method (Keeling in Fig. 1c; Miller–Tans in Fig. 1d). In both case, estimations of $\delta^{13}\text{C}_{\text{R}_s}$ were equivalent, whatever the linear regression model used (Fig. 1c and d; slope=0.99, $r=0.98$). Thus, whatever the chosen transformation method and linear regression model, there were similar estimates of $\delta^{13}\text{C}_{\text{R}_s}$.

Several outliers were excluded from the 95% confidence interval of the regression line (Fig. 1) and were characterized by a standard error $> 5\%$ (corresponding approximately to a 1‰ error here) of the estimated $\delta^{13}\text{C}_{\text{R}_s}$. Afterwards, these outliers were excluded from the dataset. Since both transformation methods and both regression models gave similar estimates of $\delta^{13}\text{C}_{\text{R}_s}$; that obtained by the Keeling

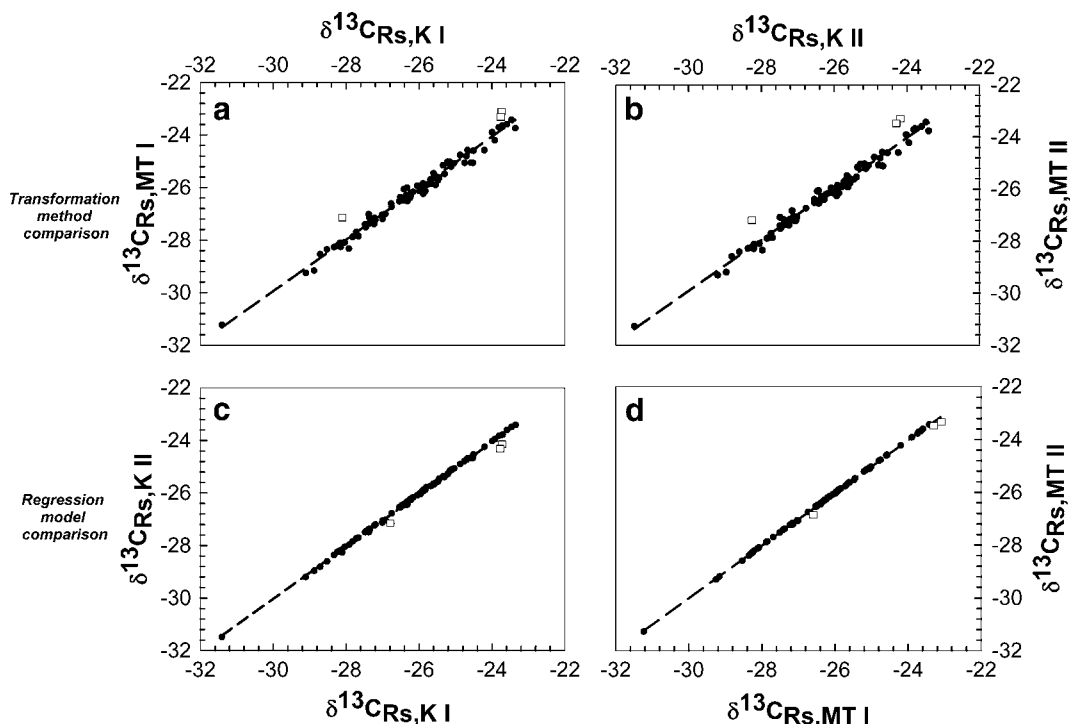


Fig. 1 Relationships between $\delta^{13}\text{C}_{\text{R}_s}$ estimated using the two transformation methods (Keeling or Miller–Tans) and the two linear regression models (Model I or model II). $\delta^{13}\text{C}_{\text{R}_s, \text{K I}}$ is $\delta^{13}\text{C}_{\text{R}_s}$ estimated using Keeling method associated to linear regression model I. $\delta^{13}\text{C}_{\text{R}_s, \text{K II}}$ is $\delta^{13}\text{C}_{\text{R}_s}$ estimated using Keeling method associated to linear regression model II.

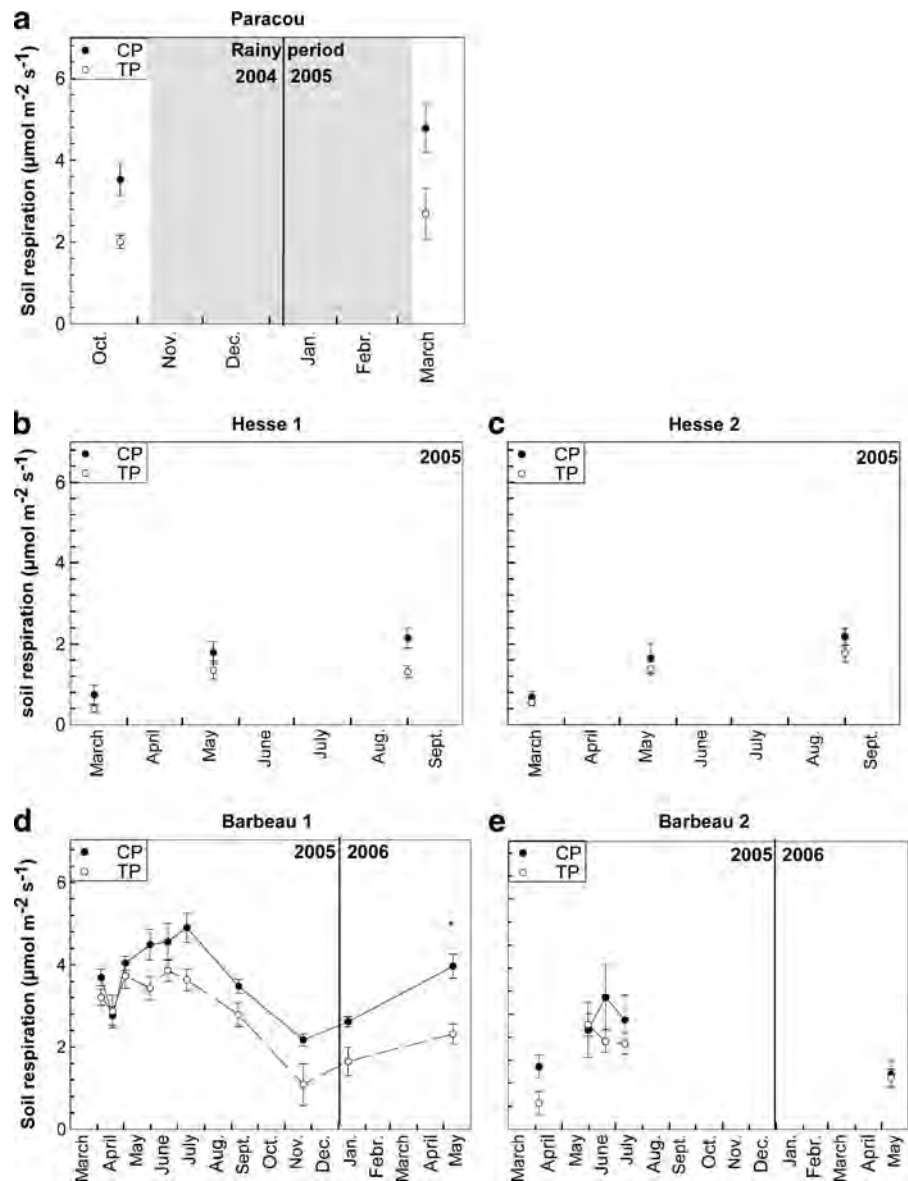
$\delta^{13}\text{C}_{\text{R}_s, \text{MT I}}$ is $\delta^{13}\text{C}_{\text{R}_s}$ estimated using Miller–Tans method associated to linear regression model I. $\delta^{13}\text{C}_{\text{R}_s, \text{MT II}}$ is $\delta^{13}\text{C}_{\text{R}_s}$ estimated using Miller–Tans method associated to linear regression model II. Open squares correspond to outliers that were excluded from the 95% confidence interval

transformation fitted with linear regression model I was further used to compare $\delta^{13}\text{C}_{\text{R}_s}$ of CP and TP.

Soil respiration

As expected, over the entire experiment period, R_s was always higher in CP than in TP at all experimental sites (Fig. 2). RM-ANOVA analysis on raw data showed that over this period trenching had a significant effect at Paracou and Barbeau ($P < 0.05$ and 0.01 , respectively), but not at Hesse site ($P < 0.3$ and 0.6 at Hesse 1 and 2, respectively).

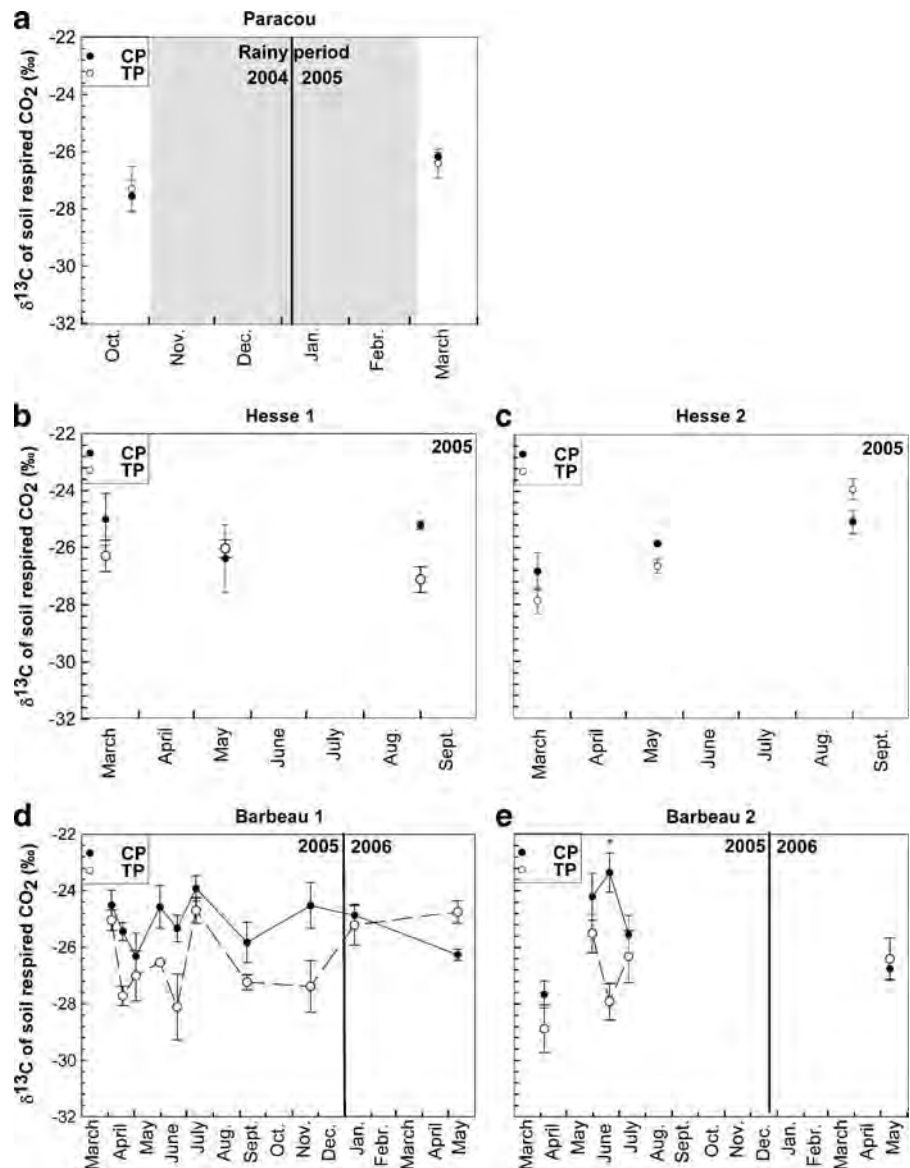
Fig. 2 Temporal variations of soil respiration in **a** Paracou ($n=4$), **b** and **c** Hesse 1 and Hesse 2 ($n=2$ to 4); **d** and **e** Barbeau 1 and Barbeau 2 ($n=8$ to 10). Control plots (CP) measurements correspond to the filled symbols and trenched plots (TP) measurements correspond to the open symbols. Error bars represent ± 1 standard error of the mean



Isotopic composition of soil CO₂ efflux

In Paracou, the difference between CP and TP was only -0.4‰ in October 2004 and $+0.3\text{‰}$ in March 2005, $\delta^{13}\text{C}_{\text{R}_s}$ being 1.3‰ less negative in March than in October (Fig. 3a). In Hesse 1 and Hesse 2, differences between $\delta^{13}\text{C}_{\text{R}_s}$ values in CP and TP ranged between -1.0 and $+1.9\text{‰}$, depending on the sampling date (Fig. 3b and c). In Barbeau 1 and Barbeau 2, the difference in $\delta^{13}\text{C}_{\text{R}_s}$ between CP and TP also showed a temporal variability with the $\delta^{13}\text{C}_{\text{R}_s}$ in TP being most often lower than CP (Fig. 3d and e).

Fig. 3 a Temporal variations of soil respired $\delta^{13}\text{C}$ - CO_2 in (a) Paracou ($n=4$), b and c Hesse 1 and Hesse 2 ($n=2$ to 4; d and e Barbeau 1 and Barbeau 2 ($n=1$). Control plots (CP) measurements correspond to the filled symbols and trenched plots (TP) measurements correspond to the open symbols. At Paracou and Hesse sites, error bars represent ± 1 standard error of the mean. At Barbeau site, error bars correspond to ± 1 standard error of the estimated $\delta^{13}\text{C}$



The difference between CP and TP ranged from -1.5 to $+4.5\%$. However, at each site, these differences were not significant, except at the end of April 2005 in Barbeau 1, and in mid-June in Barbeau 2.

$\delta^{13}\text{C}$ of bulk organic matter

Mean $\delta^{13}\text{C}$ of root, litter and soil bulk organic matter are reported in Table 2 (ROM, LOM and SOM, respectively). At all sites, ROM and LOM were significantly different from SOM ($P < 0.05$). ROM was significantly different from LOM at Paracou and Hesse ($P < 0.01$).

Discussion

Partitioning soil respiration into autotrophic and heterotrophic components is necessary to analyze the response of soil respiration to disturbances or changes in climate; however, most available methods are based on root exclusion and have strong potential drawbacks (Balesdent and Mariotti 1996; Ngao et al. 2007). The usefulness of stable isotope signatures for partitioning autotrophic and heterotrophic components has been recognized (Kuzaykov 2006) but it is still scarcely used (Rochette et al. 1999) and its applicability to forest ecosystems not tested. In such

Table 2 Mean (\pm SE) isotopic composition of root (ROM), litter (LOM) and soil (SOM) bulk organic matter, at Paracou ($n=7$), Hesse ($n=3$), and Barbeau ($n=3$)

Site	plot	Root organic matter (ROM)	Litter organic matter (LOM)	Soil organic matter (SOM)
Paracou	Mixed forest	-29.4 \pm 0.3	-30.9 \pm 0.3	-28.6 \pm 0.1
Barbeau 1	Oak high forest	-28.2 \pm 0.2	-28.2 \pm 0.2	-27.0 \pm 0.3
Barbeau 2	Oak forest with coppiced hornbeam	-28.1 \pm 0.3	-28.1 \pm 0.2	-27.0 \pm 0.3
Hesse 1	Beech high forest	-27.4 \pm 0.2	-29.0 \pm 0.2	-26.4 \pm 0.1
Hesse 2	Mixed beech forest	-28.0 \pm 0.1	-29.5 \pm 0.2	-26.9 \pm 0.2

approaches, a single isotope linear-mixing model that is based on mass conservation equations is commonly applied (Balesdent and Mariotti 1996) and reported here:

$$\frac{Ra}{R_s} = \frac{(\delta_{R_s} - \delta_h)}{\delta_a - \delta_h} \quad (4)$$

$\frac{Ra}{R_s}$ is the contribution of the autotrophic component of soil respiration; δ_a and δ_h are the respective isotopic composition of autotrophic and heterotrophic sources; and δ_{R_s} is the isotopic composition of R_s .

Applying this equation requires accurate estimates of the isotopic composition of soil respiration; differences in stable C-isotopic composition between total soil respiration and its heterotrophic components; and contrasted isotopic compositions of the different sources. These requirements are evaluated below.

Towards accurate estimates of the isotopic composition of soil CO₂ efflux

The use of the Keeling or Miller–Tans transformation methods and the linear regression models I or II to derive the isotopic signature of a respiratory flux are still debated. Some authors recommend model II (Pataki et al. 2003) while others state that only model I provides an unbiased estimate of the isotopic signature of a respiratory flux (Zobitz et al. 2006). In this study, both transformation methods and both regression models led to very similar estimates. This was partly due to high r values and the large [CO₂] ranges used to establish the relationship between [CO₂] and $\delta^{13}C_{CO_2}$. A large [CO₂] range reduces the effect of low [CO₂] values on the estimation of regression parameters (Pataki et al. 2003; Sokal and Rohlf 1995). The comparison of the different methods

also showed that they did not converge when the standard error of $\delta^{13}C_{R_s}$ estimate was $> 5\%$ of the estimated value. This convergence could be a criterion to evaluate accuracy of $\delta^{13}C_{R_s}$ estimates.

Evaluating the difference of stable C-isotope composition between total soil respiration and its heterotrophic component

R_s measured in CP and TP in Paracou are in the range found in tropical forests (Buchmann et al. 1997; Chambers et al. 2004; Epron et al. 2004b). Those measured in Hesse and Barbeau are in agreement with previous studies of temperate forests (Boone et al. 1998; Buchmann 2000; Epron et al. 2001). Over the year, R_s in CP were higher than in TP at all sites, but differences were only significant in Paracou and Barbeau. At Hesse, the decrease in R_s by trenching was not significant, probably because of the small number of replicates; only three dates of measurements were considered in this study. When considering the whole set of measurements made on these plots (every 2 weeks from March 2004 to May 2005), the difference in R_s between TP and CP were significant (Ngao et al. 2007). The differences were consistent with previous reports (Bowden et al. 1993; Epron et al. 2001; Lalonde and Prescott 2007; Subke et al. 2006). This highlights that the CO₂ release during the decomposition of severed roots only partly compensates for decreased respiration due to the removal of root respiration. Therefore trenching efficiently suppressed root respiration.

The number of replications in isotope data was constrained by cost and the time required for $\delta^{13}C_{R_s}$ measurements. Whether the studied forest ecosystem was temperate or tropical, the $\delta^{13}C$ of soil respired CO₂ ($\delta^{13}C_{R_s}$) of CP were consistent with the literature. $\delta^{13}C_{R_s}$ ranges from -28 to -21‰ (Bhudinperpal-Singh

et al. 2003; Ekblad and Hogberg 2001; Fessenden and Ehleringer 2003; Mortazavi et al. 2005; Ngao et al. 2005; Steinmann et al. 2004) and from -28 to -26‰ (Buchmann et al. 1997; Salimon et al. 2004), in temperate and tropical forests, respectively.

The comparison of $\delta^{13}\text{C}_{\text{Rs}}$ measured in control and root exclusion (trenching) conditions has never been reported. This difference of $\delta^{13}\text{C}_{\text{Rs}}$ between CP and TP plots could be used to approximate the difference ($\delta_{\text{Rs}}-\delta_{\text{h}}$) in Eq. 4. Here, despite the difference of stable C-isotope composition observed *in vitro* between root and microbial CO_2 (Andrews et al. 1999; Bowling et al. 2008; Tu and Dawson 2005), there were no pronounced differences between CP and TP, in agreement with observations of Subke et al. (2004) on girdled plots. This lack of marked difference in $\delta^{13}\text{C}$ between TP and CP is unlikely to be due to respiration of severed roots. The delay between trenching and measurements was very variable among sites and soil respiration was already reduced by 12–43% in TP without correcting for severed root decomposition. The difference of $\delta^{13}\text{C}_{\text{Rs}}$ between CP and TP could have been significant with more replications ($n=20$, standard method based on the normal distribution of differences; Sokal and Rohlf 1995). However, the difference would remain small, showing that the stable C-isotopic compositions of CO_2 from the autotrophic and heterotrophic components of soil respiration are likely to be similar. Consequently, measuring $\delta^{13}\text{C}_{\text{Rs}}$ in— and outside trench plots likely provides no additional information, beyond measuring R_{S} , to estimating the relative contribution of R_{a} and R_{h} to R_{S} . Nevertheless, although this observation is a worthy methodological point on the use of $\delta^{13}\text{C}$ in root exclusion studies, further studies are needed. Indeed, the decomposition of root organic matter added by trenching may smooth $\delta^{13}\text{C}_{\text{Rs}}$ differences between the CP and the TP plots. On another hand, trenching is known for modifying soil moisture in the TP plot, which could affect organic matter decomposition. At Barbeau sites, no correlation was found between $\delta^{13}\text{C}_{\text{Rs}}$ and soil moisture throughout the season. Moreover, small differences were also observed during girdling experiments where soil moisture was not affected by the treatment (Bhudinperpal-Singh et al. 2003; Högberg et al. 2001; Subke et al. 2004). Therefore, soil moisture modification may have little influence on $\delta^{13}\text{C}_{\text{Rs}}$ differences between TP and CP plots.

Evaluating the difference of stable C-isotope composition between autotrophic and heterotrophic sources

At all sites, $\delta^{13}\text{C}_{\text{Rs}}$ was higher than $\delta^{13}\text{C}$ for any kind of organic matter, as frequently reported (Buchmann et al. 1997; Ekblad and Hogberg 2001; Fessenden and Ehleringer 2003; Mortazavi et al. 2005; Ngao et al. 2005). These discrepancies between soil CO_2 efflux and bulk organic matter are considered as apparent isotopic fractionation underlying several actively studied mechanisms that are far beyond the scope of this study. Assuming similar fractionations for all respiratory sources, the difference between ROM and either SOM or LOM could be used to approximate the difference ($\delta_{\text{a}}-\delta_{\text{h}}$) in Eq. 4. This approximation could also be done by the difference in stable C-isotope composition between root and soil respired CO_2 measured *in vitro* (Tu and Dawson 2005). In any case, this difference would be small.

Conclusion

In the present study, we showed that the $\delta^{13}\text{C}$ of soil CO_2 efflux was not significantly affected by root exclusion in three C_3 ecosystems. This result is in agreement with the small $\delta^{13}\text{C}$ differences observed between bulk organic matter of root, litter and soil. Applying the differences ($\delta_{\text{Rs}}-\delta_{\text{h}}$) and ($\delta_{\text{a}}-\delta_{\text{h}}$) estimated above in Eq. 4 would lead to large uncertainties in the partitioning of soil respiration (Phillips and Gregg 2001; 2003); because differences are small despite their potential statistical significance. Therefore, the lack of marked difference would greatly limit the applicability of stable C-isotopes as a tool for separating R_{a} and R_{h} in C_3 ecosystems. However, seasonal changes in $\delta^{13}\text{C}_{\text{Rs}}$ and in the difference in $\delta^{13}\text{C}_{\text{Rs}}$ between CP and TP probably reflects temporal changes in the isotopic composition of available substrates for respiration, especially for roots and microbes in the rhizosphere that rely on recently assimilated carbon. The analysis of this temporal signal in soil respiration would be an opportunity to quantify the contribution of recently assimilated carbon to soil respiration, but will require frequent or even continuous measurements of soil respiration and its isotopic signature, certainly by means of ^{13}C labeling.

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Validation d'un tableau de bord d'indicateurs sur un réseau national de fermes en grande culture et en viticulture pour diagnostiquer la qualité biologique des sols agricoles

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Résumé

Dans un contexte de transition agroécologique, le projet Agrinnov a permis de valider un tableau de bord d'indicateurs analytiques de la qualité biologique des sols agricoles permettant aux agriculteurs d'appréhender l'impact de leurs pratiques. Le projet Agrinnov a amené des chercheurs et des agriculteurs à collaborer. En parallèle du tableau de bord (comprenant des indicateurs de faune du sol, de microbiologie et agronomiques), des formations sur la biologie des sols ont été élaborées ainsi qu'un mode opératoire de transfert. Les formations et le tableau de bord ont été déployés sur un réseau national de plus de 250 fermes en grande culture et en viticulture. D'un point de vue scientifique, il a été démontré que moins de 10% des parcelles testées étaient dans un état critique en termes de biologie du sol ce qui est encourageant sur la qualité des sols agricoles, même si il faudrait développer leur surveillance sur un plus grand nombre de fermes et de systèmes de production afin d'avoir un diagnostic plus exhaustif et représentatif. D'un point de vue opérationnel, plus de 97% des agriculteurs ont suivi le projet jusqu'au bout avec un fort investissement de leur part. Par conséquent, le projet Agrinnov a fait la démonstration opérationnelle de l'application et de l'appropriation par les agriculteurs des nouveaux outils de diagnostic de la qualité des sols émanant directement de la recherche,

Mots-clés : diagnostic biologique, sols agricoles, agroécologie, formation

Abstract: Biological indicators of agricultural soil quality

Within a context of agroecological transition, the Agrinnov project validated a set of indicators of the biological quality of agricultural soils to enable farmers to grasp the impact of their practices. Agrinnov project brought researchers and farmers to work together. To accompany the set of indicators of soil fauna, microbiology and agronomy, training sessions on soil biology have also been implemented together with an appropriate transfer procedure. The training sessions and the set of indicators were spread on a national network of more than 250 field-crop and viticulture farms. From an operational viewpoint, more than 97% of the farmers invested heavily in the project and stayed involved throughout

the project. From a scientific viewpoint, less than 10% of the tested plots were assessed to be in a critical state in terms of soil biology, which is encouraging on agricultural soil quality, even though the monitoring should be developed on a broader number of farms and production systems in order to have a more exhaustive and representative diagnosis. Thus the AgrInnov project demonstrated the application and adoption by the farmers of new research-based tools for diagnosing soil quality.

Keywords: biological diagnosis, agricultural soil, training, agroecology

Introduction-objectifs du projet.

Le pilotage de la qualité des sols au sein des systèmes de production agricole français est essentiellement basé sur un suivi des caractéristiques physiques (état structural...) et chimiques (pH, réserve en N, P, K, quantité et type de matière organique ...). Or, il n'est plus à démontrer que les organismes vivants du sol jouent un rôle fondamental dans son fonctionnement et plus largement dans certains services qui peuvent intéresser les productions agricoles (fertilité biologique, état sanitaire, dégradation des polluants...). De même, le concept d'assurance écologique démontre une relation positive entre la biodiversité et la productivité primaire ainsi qu'entre la biodiversité et la stabilité (durabilité) des écosystèmes (Loreau et al., 2000). Par ailleurs, le sol représente la 3^{ème} frontière biotique avec sa biodiversité qui correspond à 25% de la biodiversité totale (Maron et al., 2011). Parmi les organismes du sol, les macro-organismes sont considérés comme des ingénieurs de l'écosystème qui vont conditionner le biotope des autres organismes de la faune et microorganismes, modifiant ainsi les cycles biogéochimiques (Barot et al., 2007). Les microorganismes représentent quant à eux les organismes les plus diversifiés d'un point de vue taxonomique et fonctionnel (Bouchez et al., 2016) et sont les acteurs clés de la plupart des cycles biogéochimiques. Entre les deux, les organismes de la microfaune et mésofaune jouent aussi un rôle complémentaire dans l'évolution de la matière organique et les cycles des nutriments (Cortet et al., 1999).

Dans un contexte agricole en pleine mutation, il apparaît aujourd'hui essentiel de se doter d'outils de surveillance de la qualité du sol permettant d'appréhender l'impact des pratiques (labour, pesticides, rotation, fertilisation) sur son fonctionnement biologique, et les services qu'il rend pour la production agricole. En effet, les organismes vivants du sol (faune du sol, bactéries, champignons) jouent un rôle fondamental dans son fonctionnement : dynamique des matières organiques et cycle du carbone et de l'azote, biodisponibilité des éléments nutritifs, dégradation de polluants organiques, rétention de polluants métalliques, action sur la structure des sols, etc. Ces communautés biologiques sont susceptibles de traduire l'ensemble des stress environnementaux de leur milieu et, par la précocité de leurs réactions, ils apparaissent donc comme de bons indicateurs de l'évolution des sols (Ranjard et al., 2010).

C'est dans ce contexte qu'est né le projet AgrInnov (CASDAR 2012-2015) qui visait à valider l'utilisation d'indicateurs biologiques de la qualité du sol, tout en créant un mode opératoire de transfert et des formations. Il devait aussi permettre de poser les fondations d'un réseau de veille à l'innovation agricole (REVA) articulé notamment autour du thème de l'impact des pratiques agricoles sur la vie biologique des sols. Celle-ci serait mesurée par des bioindicateurs ciblant les lombrics, les nématodes et les microorganismes, et couplés à des indicateurs agronomiques (état structural et physico-chimie du sol, dégradation de la matière organique).

L'équipe projet a réuni les experts nationaux des indicateurs biologiques et agronomiques les plus à-mêmes de répondre aux attentes des agriculteurs. Une des originalités et LE challenge du projet AgrInnov étaient de faire travailler ensemble les chercheurs avec les agriculteurs, chacun restant expert dans son domaine. Les agriculteurs avaient pour mission de décrire leurs besoins, de mesurer, et de trouver parmi les outils qui leurs seraient proposés, ceux qu'ils accepteraient de tester. Les chercheurs avaient pour mission de faire correspondre aux besoins de mesure des agriculteurs, des outils déjà

validés par la recherche, et de créer le contexte et l'accompagnement appropriés (formation, mode opératoire, documents de restitution) pour que les agriculteurs puissent les tester et se les approprier.

Le **choix des bioindicateurs retenus** dans le cadre de ce projet était guidé par leur aspect opérationnel et l'existence d'un référentiel d'interprétation spécifique. Il a donc été développé un véritable diagnostic de la qualité biologique et agronomique des sols agricoles. En outre, pour faciliter la lecture des informations récoltées, l'équipe projet a développé des « indicateurs de synthèse ». Ces derniers combinent les valeurs issues de différents indicateurs élémentaires complémentaires, dont l'assemblage permet la caractérisation et le diagnostic agronomique des grandes fonctions du sol, comme le patrimoine biologique / assurance écologique, et la fertilité biologique.

En parallèle du tableau de bord analytique, des **formations sur la biologie des sols** ont été mises en place afin d'accompagner l'appropriation des indicateurs biologiques. Celles-ci intégraient des aspects variés couvrant des notions de base en biologie des systèmes terrestres, la description des différents indicateurs disponibles, ou encore des études d'impact des pratiques agricoles sur la biologie du sol et les fonctions qu'elle porte. L'ensemble « tableau de bord et formation » a été déployé sur le réseau AgrInnov, créé dans le cadre du projet et constitué de fermes agricoles en grande culture (125) et en viticulture (123). AgrInnov a permis d'établir un contexte d'échange des savoirs dans lequel les agriculteurs pourront plus facilement mettre de côté une vision essentiellement productiviste de la parcelle, et s'approprier une vision d'un écosystème « piloté de manière à fournir durablement diverses catégories de biens et de services précisément qualifiés » (« Projet Transition Agroécologique pour la France » Ministère de l'agriculture, 2015).

1. Constitution et rôle du Groupe de Travail Mixte (GTM)

Une des originalités et le grand challenge du projet AgrInnov étaient de faire travailler directement les chercheurs et les agriculteurs. Les GTM étaient donc les rencontres lors desquelles les experts, chercheurs et formateurs développaient une formation et un tableau de bord sur la biologie des sols, qui devaient correspondre au mieux aux attentes et besoins des agriculteurs. Ainsi, les agriculteurs échangeaient avec les chercheurs lors des nombreuses phases d'interactions organisées dans le projet (formation, échantillonnage, rendu de résultats) afin d'identifier les outils les plus opérationnels pour eux et aussi les pratiques agricoles les plus éco efficaces au vu des résultats d'analyse.

L'objectif des GTM était d'impliquer la plus grande diversité possible d'agriculteurs. Les groupes étaient donc basés sur le volontariat des agriculteurs. Les GTM ont permis notamment d'optimiser le nombre d'indicateurs du tableau de bord en éliminant les moins opérationnels mais aussi d'améliorer certaines techniques d'échantillonnage sur le terrain pour les rendre plus opérationnelles. Les agriculteurs ont été aussi fortement critiques et structurants sur la mise en place de la formation sur la biologie des sols agricoles pour qu'elle corresponde au plus près aux attentes de la profession.

2. Inventaire et sélection des indicateurs

2.1 Les indicateurs élémentaires

Les bioindicateurs retenus dans le cadre de ce projet se basent sur des mesures de la faune (nématodes et lombrics) ainsi que des microorganismes du sol (bactéries et champignons). Ces organismes sont reconnus pour jouer un rôle essentiel dans le bon fonctionnement biologique des sols et la durabilité des agrosystèmes. Au sein de ces deux groupes, le choix des indicateurs a été fait sur la base de l'expertise des unités de recherche impliquées (INRA, Université). Les critères d'évaluation des indicateurs étaient :

- d'être validés scientifiquement (sensibilité, fiabilité, spécificité) via des programmes de recherche (ADEME Bioindicateur I et II, ANR ECOMIC-RMQS, ADEME RMQS-BIODIV, EU ENVASSO, EU Ecofinder...),
- de disposer d'un référentiel d'interprétation pour la viticulture et les grandes cultures afin d'identifier la gamme de variation normale en fonction des types pédo-climatiques et d'usages des sols et ainsi permettre de diagnostiquer précocement l'impact des pratiques agricoles ainsi que les modifications du fonctionnement biologique des sols,
- d'intégrer des fonctions biologiques supportant des services rendus par les agrosystèmes (fertilité, réduction des GES, protection des cultures, dépollution, durabilité...),
- d'être mesurables à un coût économique abordable, facilement opérationnels sur le terrain et au laboratoire et donc utilisables et interprétables par les agriculteurs et les agents du développement rural,
- d'avoir obtenu la validation du GTM constitué d'experts et d'agriculteurs.

A la suite de la sélection effectuée, la liste finale des indicateurs retenus pour constituer le tableau de bord analytique AgrInnov (Figure 1) est la suivante :

- Abondance lombricienne totale
- Abondance des 4 groupes fonctionnels de lombriciens (épigé, épi-anécique, anécique, endogé)
- Diversité et structure taxonomiques des communautés lombriciennes
- Biomasse moléculaire microbienne
- Rapport densité champignons/densité des bactéries
- Diversité taxonomique des bactéries et des champignons
- Abondance des nématodes libres
- Diversité taxonomique des nématodes
- Abondance des nématodes phytoparasites
- Indice de structure des nématodes
- Indice d'enrichissement des nématodes
- Structure du sol (par un test bêche)
- Caractéristiques physico-chimiques des sols
- Teneur en polluants métalliques (Cu, Ni, Pb...)
- Dégradation de la matière organique (par la méthode du litter-bag)

Grâce à la présence d'un référentiel d'interprétation spécifique à chaque type d'indicateur élémentaire, un véritable diagnostic de la qualité biologique et agronomique des sols agricoles a donc pu être développé.



Figure 1 : Liste des outils et indicateurs biologiques et agronomiques constituant le tableau de bord du projet AgrInnov

2.2 Les indicateurs de synthèse©

En parallèle, l'équipe projet a développé des **indicateurs de synthèse©** qui permettent d'avoir une vision plus synthétique de l'information fournie par l'ensemble des indicateurs. Ces indicateurs de synthèse intègrent différents indicateurs élémentaires agronomiques et biologiques complémentaires pour diagnostiquer des grandes fonctions du sol d'intérêt agronomique. A ce jour, deux indicateurs de synthèse ont été développés :

- **l'indicateur patrimoine biologique / assurance écologique**, qui renseigne sur la capacité d'un sol à héberger une forte abondance et diversité d'organismes vivants mais aussi sur les équilibres biologiques entre ces organismes.
- **l'indicateur fertilité biologique**, qui renseigne sur la capacité d'un sol à dégrader la matière organique endogène ou apportée par les pratiques culturales (amendements, résidus de culture).

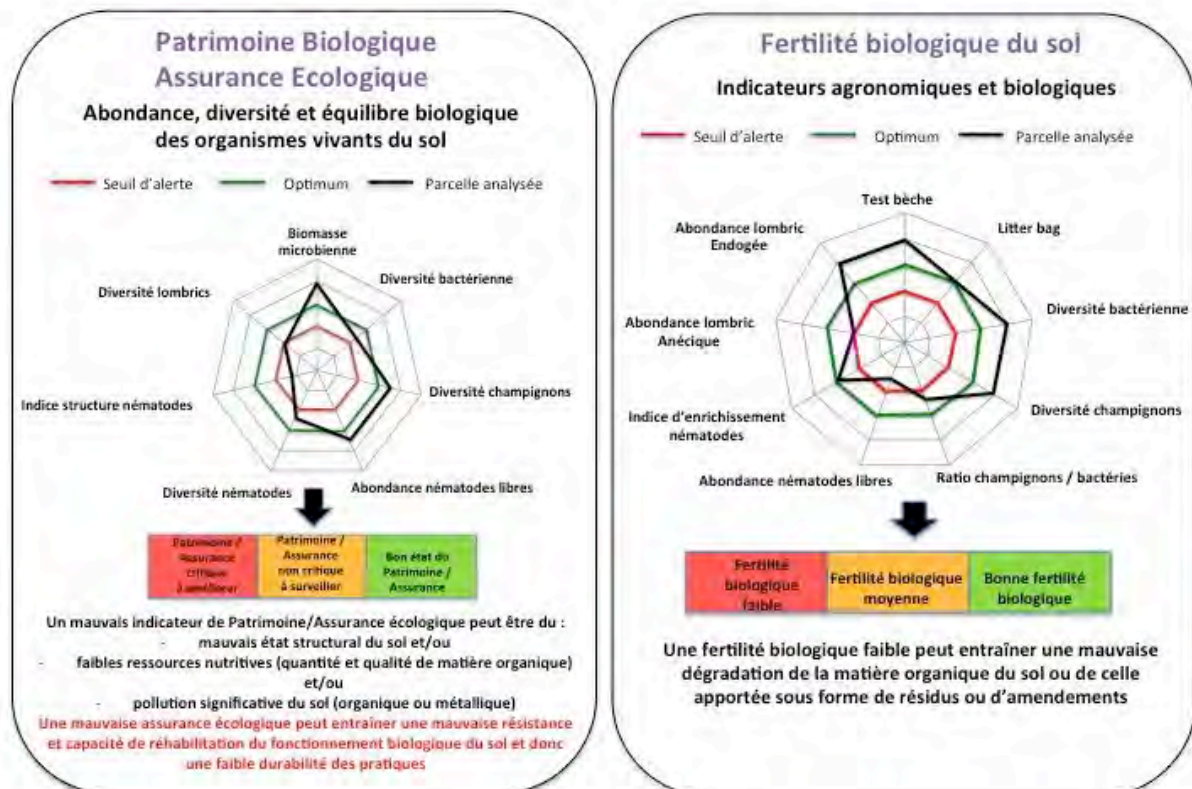


Figure 2 : Indicateurs de synthèse© de la qualité biologique des sols.

3. Modalités de la mise en place au sein du réseau

3.1 Mise en place d'un réseau national de fermes : le réseau AgrInnov.

Après validation par le GTM de la liste d'indicateurs proposés dans l'étape 2, ceux-ci ont été déployés au sein du réseau AgrInnov. La stratégie développée était de dispenser dans un premier temps la formation sur la biologie du sol, et dans un second temps de laisser les agriculteurs réaliser les prélèvements de leur sol. Les résultats d'analyse regroupés dans un tableau de bord agricole leur sont ensuite retournés. Il a donc été décidé :

- de créer des groupes d'agriculteurs exploitant des fermes proches géographiquement mais développant des pratiques différentes (pour évaluer aux mieux l'effet de pratiques différentes dans un pédoclimat comparable) ;
- de ne traiter qu'une seule parcelle par agriculteur ;
- de réunir 10 à 15 fermes par groupe.

Il a ainsi été constitué 10 groupes d'agriculteurs et 10 groupes de viticulteurs représentant respectivement 125 et 123 parcelles, dont la Figure 3 présente la répartition à l'échelle du territoire. Ce réseau que nous avons baptisé « Réseau AgrInnov » a été mis en place et coordonné grâce aux acteurs du développement agricole (CA, ITA, APAD, groupements agricoles, ...)

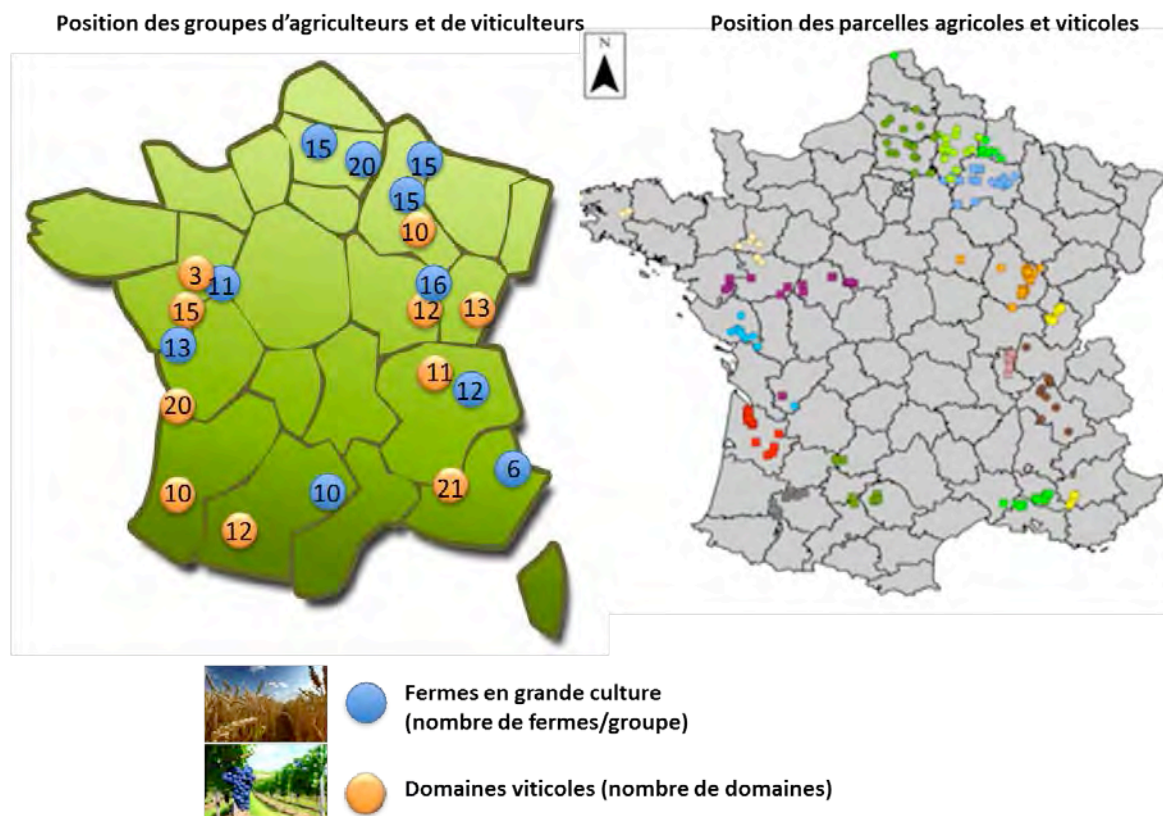


Figure 3 : Réseau « AgrInnov » de fermes en grandes cultures et de domaines viticoles

Ce réseau affiche une surreprésentation de certains systèmes de production au regard de leur place réelle dans le paysage agricole national, comme les exploitations en agriculture biologique ou agriculture de conservation des sols. Ceci peut s'expliquer par le fait que nous avons recherché l'adhésion d'agriculteurs et de viticulteurs volontaires, ce qui rassemble les plus concernés par l'impact

des pratiques sur la qualité des sols qui automatiquement, sont souvent les plus innovants dans leur domaine.

3.2 *Elaboration d'un guide d'échantillonnage*

Un groupe d'experts a été constitué pour réaliser un guide d'échantillonnage par type de production (grande culture et vigne). Ce choix est motivé par la spécificité de la configuration des rangs de vignes par rapport aux grandes cultures. Le groupe réunissait un expert de chaque indicateur, un expert en viticulture, et un en grande culture. Le guide d'échantillonnage détaille :

- les conditions nécessaires et la période idéale pour les prélèvements,
- les dispositifs d'échantillonnage (localisation relative des différents types de prélèvements),
- le matériel nécessaire et la procédure détaillée de réalisation de chaque type de prélèvement
- les conditions d'expédition et de conditionnement des échantillons.

Tous les indicateurs élémentaires sont échantillonnés directement sur les parcelles agricoles. Certains sont analysés immédiatement sur le terrain (ex. comptage de vers de terre, test bêche) tandis que d'autres sont analysés en laboratoire spécialisé (ex. comptage de nématodes par observation microscopique, caractérisation de la biomasse et de la diversité microbienne par des outils de biologie moléculaire basés sur la caractérisation de l'ADN du sol). Une séquence technique et logistique rigoureuse a été développée pour organiser l'échantillonnage, l'envoi d'échantillons de sols et d'échantillons biologiques (par les agriculteurs), le stockage de ces échantillons et leur analyse et le référencement des résultats obtenus dans une base de données.

3.3 *Stratégie de mise en place au sein du réseau*

L'ensemble des outils développés (formation, tableau de bord et guide) a été testé auprès des fermes du nord-ouest de la France, soit une quarantaine au total. Les suggestions émises à l'issue de ce test ont alors été prises en compte par les chercheurs et les formateurs pour améliorer la formation mais aussi les procédures d'échantillonnage au terrain et l'accompagnement dans l'interprétation des résultats. De la même manière et dans le même temps, ces tests grandeur nature ont permis à l'équipe projet de réaliser une analyse critique des outils et des moyens de transfert qu'elle avait développés. Elle a ainsi pu améliorer l'organisation de ses actions, qu'il s'agisse :

- Du contenu et de l'organisation des formations,
- De la logistique de l'échantillonnage,
- Du contenu, des documents, et de la présentation des sessions de restitution des résultats.

Après le franchissement de cette étape cruciale, l'ensemble des outils de diagnostic finalisés a été diffusé au reste du réseau national, soit 248 parcelles réparties dans les quarts de France Nord Est, Sud Est et Sud Ouest.

4. **Formation, Restitution et Transfert**

Cette étape avait pour objet d'assurer le transfert et l'utilisation des outils développés dans le cadre du projet mais hors du réseau Agrinnov, vers les utilisateurs agriculteurs, conseillers/techniciens agricoles, étudiants et laboratoires commerciaux mettant en œuvre des analyses biologiques. Le transfert vers les étudiants ingénieurs et en licence professionnelle « production végétale » a été réalisé partiellement par les établissements d'enseignement partenaires d'AgriInnov (ISARA, ESA, AgroSup Dijon). Le transfert aux agriculteurs et conseillers/techniciens a pu être largement mis en œuvre avec des outils de formation harmonisés. Ces formations étaient destinées à fournir les connaissances nécessaires pour

(i) mieux connaître la vie biologique des sols et les indicateurs retenus, (ii) prélever les échantillons, et (iii) savoir interpréter ses résultats par rapport à un référentiel et comprendre les variations observées.

L'ingénierie des formations a été pensée en collaboration étroite avec les agriculteurs dans le cadre des GTM, afin de garantir la meilleure adéquation à leurs besoins. Ces interactions ont permis de valider le cahier des charges complet des formations sous la forme d'un kit de formation. La formation dure une journée et demie, et la première journée est divisée en deux parties :

- la première partie théorique est dispensée en salle. Elle présente aux agriculteurs la biologie du sol, les services agronomiques et environnementaux rendu aux productions agricoles, et comporte des exemples de l'impact des pratiques agricoles. Elle permet aussi la présentation technique des différents indicateurs qui constituent le tableau de bord AgrInnov et explique leur intérêt.
- la deuxième partie est composée de travaux pratiques. Elle a lieu l'après-midi sur une parcelle agricole d'un des agriculteurs stagiaires. Toutes les étapes d'échantillonnage de sols, de prélèvements biologiques (vers de terre), et de réalisation du test bêche et du litterbag, sont expliquées et réalisées. Cette partie de la formation s'appuie sur le guide d'échantillonnage élaboré dans le cadre du projet.

Les formations ont eu lieu en automne ou au printemps, périodes les plus propices à l'échantillonnage tant en termes de conditions climatiques que de disponibilité des agriculteurs. Par la suite, les agriculteurs disposaient de 4 à 6 semaines pour échantillonner sur leurs parcelles et envoyer les échantillons aux différents experts/laboratoires. En parallèle, les agriculteurs étaient invités à remplir un questionnaire sur les pratiques agricoles de la parcelle échantillonnée. Les experts ont disposé de 3 à 6 mois pour analyser les échantillons.

Une dernière demi-journée de formation est consacrée à la restitution des résultats sur les lieux de la formation. Pour cette restitution un effort particulier a été mené par l'équipe projet pour développer des fiches synthétiques présentant les résultats pour chaque indicateur élémentaire et les indicateurs de synthèse (Figure 4).

Les formations ont été coordonnées pour chaque quart de France par les partenaires locaux du projet AgrInnov :

- au Nord-Ouest : CA 49 et ESA Angers ;
- au Nord-Est : AgroSup Dijon ;
- au Sud-Est : ISARA Lyon ;
- au Sud-Ouest : IFV ;

La coordination nationale a été réalisée par l'OFSV.

Au total 20 groupes ont été formés, représentant environ 300 personnes : 248 agriculteurs et 50 invités (techniciens de chambres et de coopératives et agro fournisseurs). Au final, sur les 248 agriculteurs et viticulteurs qui ont suivi l'un de 20 ateliers de formation, 240 ont échantillonné de façon rigoureuse leur sol pour les faire analyser. 97% des agriculteurs et viticulteurs ont donc adhéré au projet jusqu'au bout. Il n'y a pas de différence significative de retour entre le réseau viticole et le réseau grande culture.

Des enquêtes de satisfaction ont été mises en place et elles montrent le réel intérêt des acteurs du monde agricole à disposer d'outils de diagnostic sur la qualité biologique des sols afin d'évaluer l'impact de leurs pratiques et la durabilité de leur production (Figure 5). Elles montrent aussi que la démarche d'AgriInnov est pertinente pour diffuser ces outils et ces nouveaux concepts au niveau des agriculteurs.

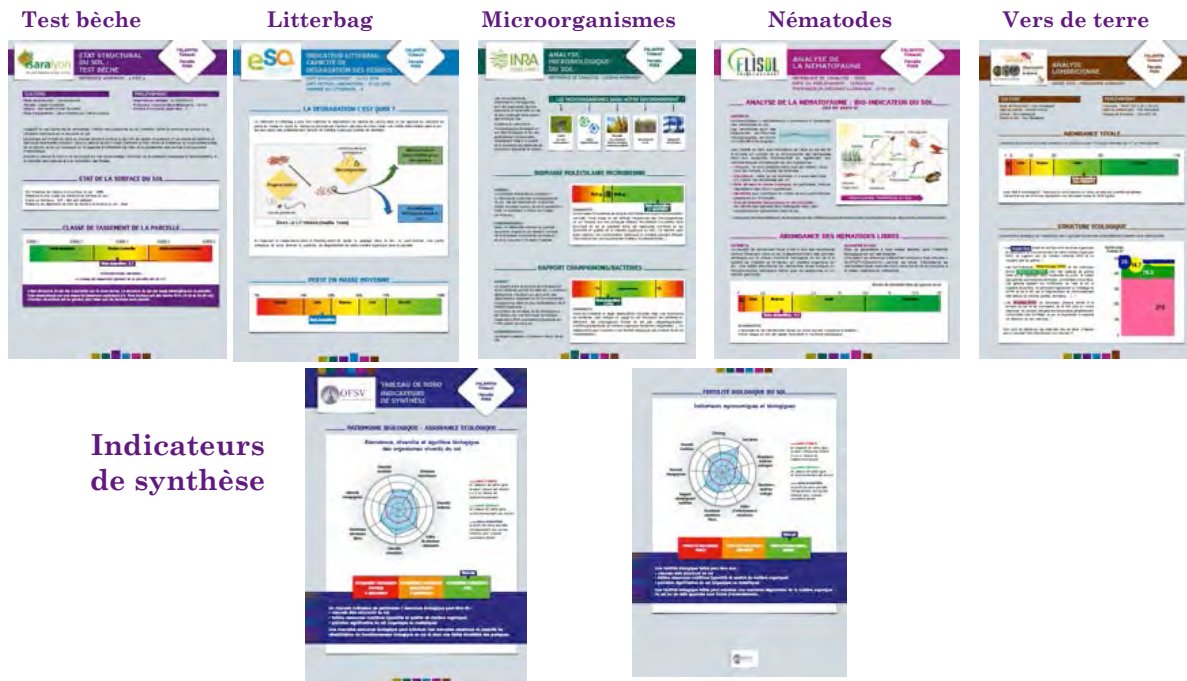


Figure 4 : Fiche individuelle de résultats.

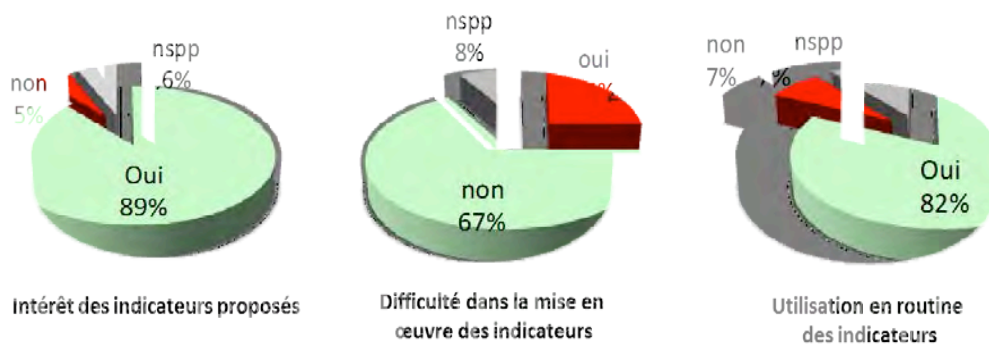


Figure 5 : Résultats des enquêtes de satisfaction des agriculteurs et viticulteurs au sujet de la formation et du tableau de bord analytique Agrlnnov

5. Analyse issue des résultats du réseau national Agrlnnov

5.1 Clusterisation des itinéraires techniques en grande culture et en viticulture du réseau de parcelles Agrlnnov

Dans le projet, les agriculteurs ont rempli des enquêtes sur leurs pratiques et leur système de production. La compilation de ces enquêtes a permis d'analyser la représentativité de certaines caractéristiques des systèmes de production du réseau Agrlnnov, (sans labour, bio, conventionnelle, ecophyto...) et de segmenter la population de parcelles en fonction des différents types d'itinéraires techniques (ITK). Une typologie mathématique de « clusterisation » a été combinée avec une typologie « à dire d'experts » et a permis de définir les grands critères de segmentation :

- Travail du sol
- Assolement et longueur des rotations : couvert, diversité des rotations

- Fertilisation et amendement : engrais chimiques, apport de matière organique endogène ou exogène (MOE)
- Protection phytosanitaire

Ce tri statistique des itinéraires techniques était indispensable pour pouvoir confronter les résultats des indicateurs biologiques et agronomiques aux pratiques des agriculteurs. Les tableaux ci-dessous présentent les typologies issues respectivement des enquêtes en grandes cultures et des enquêtes en vignes.

Grandes cultures	GC1	GC2	GC3	GC4.1	GC4.2
Travail du sol	--	-	-	+	+
MOE	--	-	+	+	-
Couvert	++	+	+	+/-	-
Phyto	++	++	++	+	+

Vignes	V1	V2	V3	V4	V5	V6
Travail mécanique	--	--	--	+	+	++
MOE	+	--	++	++	++	++
Couverture inter-rang	+	++	++	+	+	--
Phyto	++	+/-	+/-	+/-	--	+

Figure 6 : Catégories d'itinéraires techniques au sein du réseau Agrlnnov, en haut pour les grandes cultures et en bas pour les vignes.

5.2 Analyse du tableau de bord complet

Les Figures 7 et 8 représentent pour chaque groupe issu des typologies de pratiques les valeurs moyennes des paramètres des indicateurs biologiques et agronomiques.

- « rouge » pour un score moyen inférieur au seuil d'alerte ;
- « orange » pour un score compris entre le seuil d'alerte et l'optimum ;
- « vert » pour un score moyen supérieur à l'optimum.

Ces premiers résultats montrent :

- que les indicateurs élémentaires du tableau de bord sont complémentaires car ils ne donnent pas tous la même tendance au sein et entre les itinéraires techniques.
- que la sensibilité des indicateurs est différente en fonction du système de production (grande culture vs viticulture) ; pour exemple, la biomasse moléculaire microbienne est majoritairement dans le vert pour les sols de grandes cultures mais majoritairement dans le rouge pour les sols viticoles.

D'un point de vue plus général, la qualité biologique et agronomique des sols dans l'échantillon considéré est meilleure dans les sols de grande culture en comparaison des sols viticoles. Par ailleurs, ces résultats peuvent être partiellement influencés par les variations de type de sols et de climats entre les parcelles d'un même groupe d'itinéraires techniques. Toutefois, il apparaît globalement difficile de différencier les itinéraires techniques uniquement en se focalisant sur les performances des indicateurs biologiques et agronomiques.

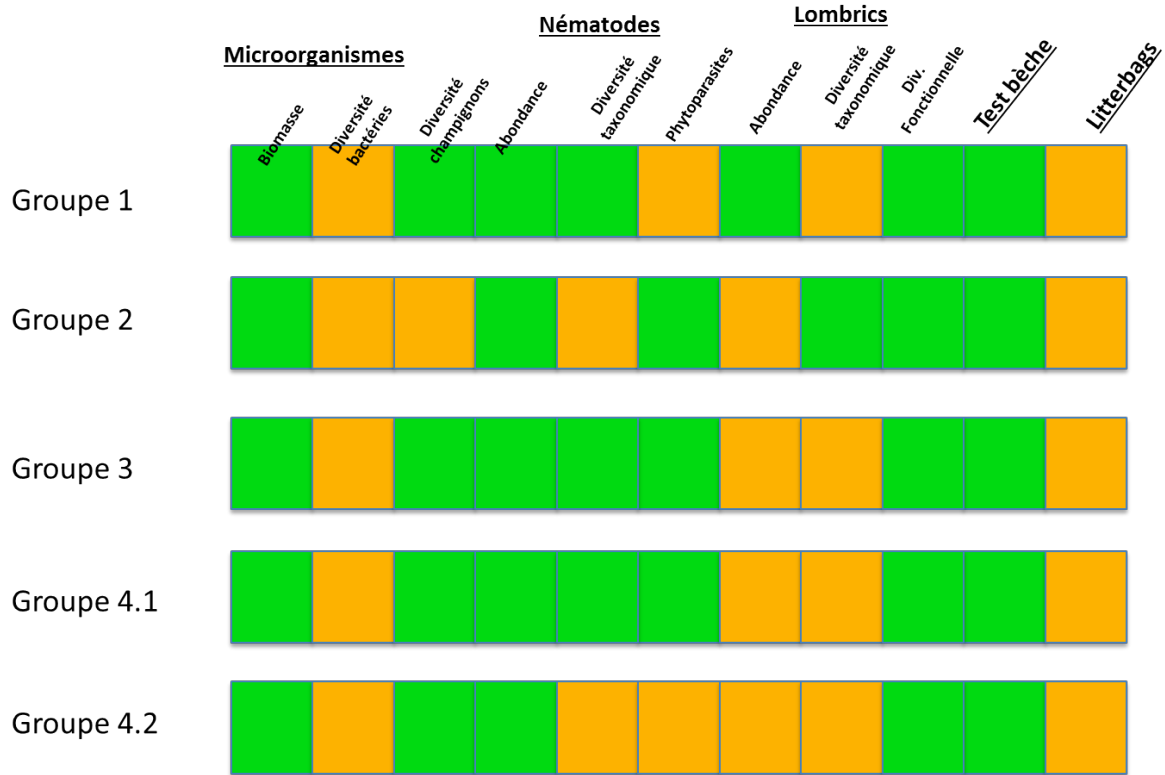


Figure 7 : Evaluation des systèmes de production en grandes cultures

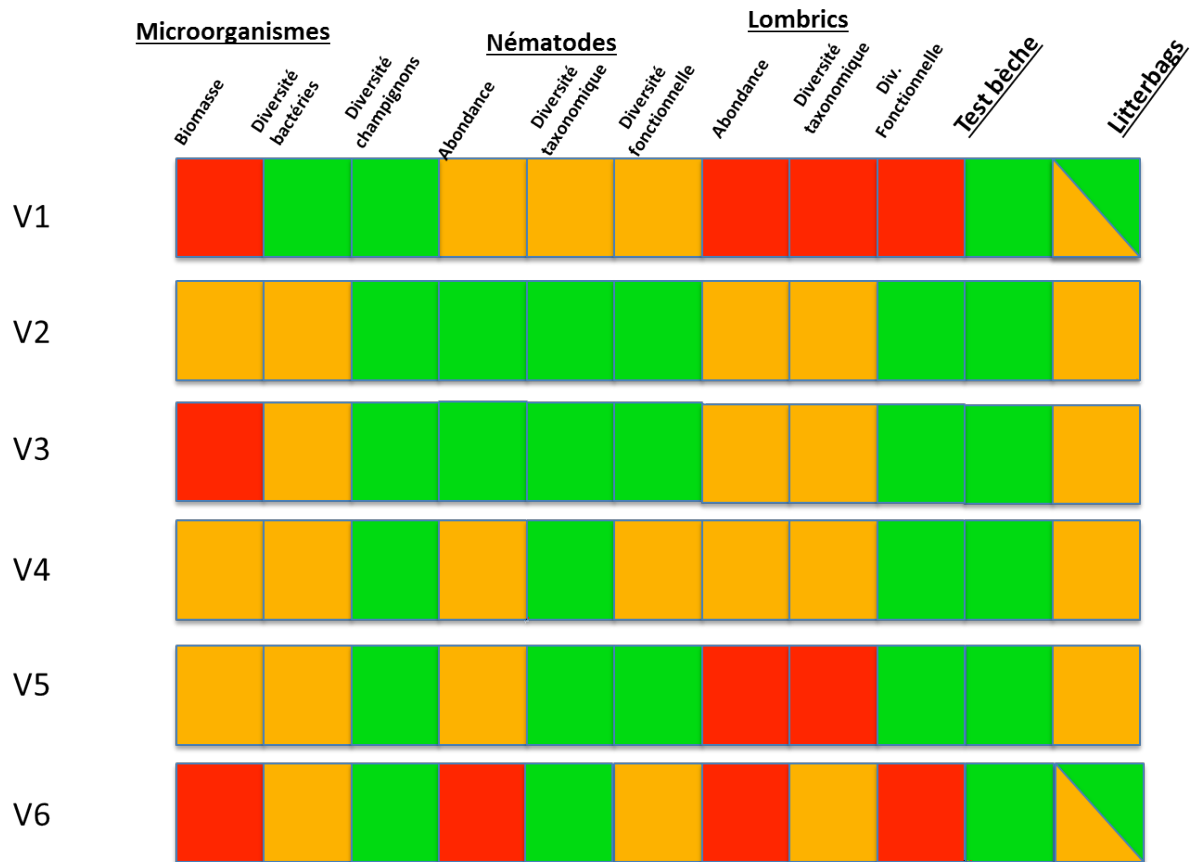


Figure 8 : Evaluation des systèmes de production en viticulture

5.3 Analyse des indicateurs de synthèse.

La distribution de l'ensemble des parcelles analysées selon le résultat des indicateurs de synthèse est présentée dans la Figure 9.

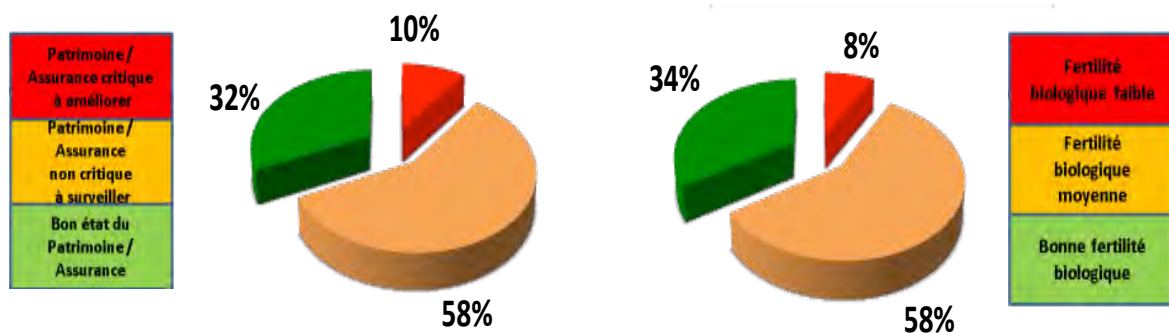


Figure 9 : Analyse des indicateurs de synthèse® sur le réseau AgrInnov.

Un des résultats marquants du projet AgrInnov est la démonstration que seulement 10% des sols des parcelles agricoles et viticoles étudiés sont dépréciés en termes de patrimoine biologique et de fertilité biologique ; ce résultat mériterait d'être consolidé en étendant l'étude sur un plus grand nombre de sols et de systèmes de production. Une analyse plus détaillée par système de production a toutefois montré que les sols viticoles sont plus altérés en termes de biologie et fonctions biologiques du sol que les sols en grande culture. Ceci peut s'expliquer par le fait que l'orientation productive initiale d'une parcelle en « grande culture » ou « vigne » est notamment dictée par le type de sol. Un autre paramètre explicatif est que les systèmes de production viticoles sont plus agressifs pour la biologie des sols et les fonctions et services qui en découlent.

6. Modalités de valorisation et extension du projet

Lors du déroulement du projet le site internet de l'OFSV (www.ofsv.org) a présenté les différentes avancées du projet et ses résultats opérationnels les plus marquants. De plus, des films sur les formations ont été réalisés et accompagnent le guide d'échantillonnage et d'analyse des différents bioindicateurs du projet. Ces films sont à ce jour finalisés et seront à disposition sur le site internet de l'OFSV qui sera réaménagé dans ce but.

Le projet AgrInnov a abouti à l'organisation des Journées de l'Innovation Agricole, (www.jiag.info). Il s'agit d'un colloque de deux jours qui s'est tenu les 2-3 novembre 2015 au centre des congrès d'Angers. Ce colloque avait pour objectif de faire le bilan des projets de recherche et de développement menés par les instituts de recherche et technique, les chambres d'agriculture, les groupements agricoles etc. afin d'avoir une meilleure connaissance de la biologie des sols agricoles et la possibilité de développer des nouveaux outils de diagnostic et de conseil adaptés en agroécologie. Ce colloque était articulé autour de la présentation du projet AgrInnov et des avancées significatives qu'il a permis d'un point de vue technique, scientifique et opérationnel. Il a réuni plus de 325 personnes : agriculteurs, étudiants, décideurs, politiques, acteurs du développement agricole et chercheurs. Il a aussi permis de voir que les entreprises d'agro fourniture sont elles aussi demandeuses de ce genre de manifestation pour comprendre plus vite le besoin des agriculteurs et faire évoluer leur offre.

D'autres modes de communication ont également été utilisés pour communiquer et vulgariser les résultats du projet AgrInnov (presse technique agricoles, presse grand public, congrès scientifiques nationaux et internationaux, manifestation agricoles etc...). Le recours à cette gamme très large de

canaux de communication a permis de toucher et de sensibiliser un public très large sur l'intérêt de la biologie du sol pour le diagnostic de l'état du sol dans un contexte d'agroécologie.

Conclusion

Le projet AgrInnov s'est terminé en juin 2015 et a fait la démonstration opérationnelle que les agriculteurs sont prêts à acquérir de nouvelles connaissances sur la biologie des sols et à modifier leurs pratiques selon ces nouvelles connaissances. Ceci n'est possible que par une interaction forte et à double sens entre les chercheurs et les agriculteurs. Par la suite, afin de pérenniser les groupes d'agriculteurs et de viticulteurs formés à l'échelle nationale, différentes initiatives régionales sont en train de voir le jour par l'intermédiaire notamment des financements FEADER et des Agences de l'eau, des contrats territoriaux, des contrats filières, de l'ADEME, des régions,....ou par l'intermédiaire de programmes comme Ecophyto. Au niveau des régions Bourgogne, PACA, Pays de Loire, Midi-Pyrénées, Languedoc Roussillon, Poitou-Charentes et Champagne-Ardenne, les chambres d'agriculture, instituts techniques ou groupements agricoles (GIEE) vont déposer des demandes de financement (FEADER, Agence de l'eau, GIEE...) pour pérenniser et étoffer les groupes de viticulteurs et d'agriculteurs mis en place. Ce réseau, initié sur la base du réseau AgrInnov, s'appelle le **REVA** (Réseau de Veille à l'Innovation Agricole). La coordination nationale de ce réseau est assurée par l'Observatoire Français des Sols Vivants (www.ofsv.org) et la coordination locale par les différents organismes impliqués dans AgrInnov (AgroSup Dijon, ISARA, IFV, ESA, CA, Univ. Rennes...). En parallèle, l'équipe d'experts AgrInnov est en train de finaliser un partenariat plus pérenne pour protéger et diffuser la formation et le tableau de bord développés dans le projet.

Dans ce contexte et à la suite d'AgrInnov, des structures de prestation ont été confortées dans leur position et d'autres ont vu le jour. La société ELISOL environnement et l'OPVT ont pu, grâce au projet, consolider leur rôle d'expert sur la faune du sol en lien avec les pratiques agricoles. De plus, l'ESA a développé une nouvelle structure (LEVAbag MD) qui permettra de diffuser une nouvelle prestation de service dans le monde agricole basée sur les litter bags. Par conséquent, les filières de formation et de diffusion du tableau du bord analytique sur les indicateurs de la biologie de sols agricoles sont initiées et consolidées grâce au projet AgrInnov. Ces filières pourront pleinement se positionner et s'exprimer dans le futur réseau REVA.

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