Variations spatio-temporelle de la microflore des sols alpins

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Variations spatio-temporelles de la microflore des sols alpins

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Préface: présentation de la démarche

Cette thèse s'inscrit dans le cadre général **de l'écologie du sol**. Plus particulièrement, elle s'est donnée pour objectif la caractérisation des **variations spatiales et temporelles des communautés microbiennes des sols alpins** en relation avec le couvert végétal *via* le développement de techniques moléculaires et d'outils d'analyses. Cette étude s'articule ainsi en trois parties principales correspondant aux chapitres du présent manuscrit.

La thématique « microbiologie environnementale » venait de naître au laboratoire, sous l'impulsion de Roberto Geremia, lors de mon arrivée au LECA (Laboratoire d'Ecologie Alpine), dans l'équipe PEX (Perturbations Environnementales et Xénobiotiques). Cette thématique vise à déterminer l'impact de changements environnementaux sur les communautés microbiennes. Le premier objectif de cette thèse a donc été de mettre en place au laboratoire des outils d'études des communautés microbiennes, et d'**optimiser** ces techniques, notamment la technique d'empreinte moléculaire **CE-SSCP** (Capillary-Electrophoresis Single-Strand Conformation Polymorphism). Dans cette optique, nous nous sommes employés à tester une série d'étapes manipulatoires (extraction d'ADN, conditions PCR, etc…) et leurs répercussions sur les profils moléculaires des communautés bactériennes, en utilisant le gène de l'ARN ribosomique 16S. Ces travaux font l'objet d'une publication de *Microbial Ecology* (Zinger et al., 2007; cf. Chapitre I). Nous avons ainsi pu mettre en évidence un effet notable du type d'ADN polymérase utilisé sur le nombre de pics observés dans les profils, et dont les répercussions s'étendent à toutes les disciplines utilisant les techniques d'empreintes moléculaires. Les résultats de cette étude sont valorisés dans une publication de *Electrophoresis* (Gury et al., 2008; Annexe A). Nous avons ensuite voulu étendre l'utilisation de la CE-SSCP aux communautés fongiques des sols, en utilisant le marqueur moléculaire ITS1 (Intergenic Transcript Spacer 1), et tester l'efficacité de cette méthode en parallèle avec une autre technique d'empreinte moléculaire, la CE-FLA (Fragment Length Analysis, homonyme de la F-ARISA). Ces travaux sont exposés dans une publication de *Journal of Microbiological Methods* (Zinger et al., 2008; cf. Chapitre I).

Les optimisations techniques décrites dans le chapitre I ont ensuite été appliquées à l'étude de la distribution des micro-organismes du sol dans des écosystèmes alpins. Les sols

de ces écosystèmes sont soumis à des froids intenses qui ralentissent la dégradation de la matière organique et constituent ainsi des stocks de carbone importants dont le devenir reste incertain dans un contexte de réchauffement climatique. Dans un premier temps, notre étude s'est restreinte à **deux habitats contrastés par leur enneigement**, l'un avec de forts contrastes thermiques en raison d'un enneigement faible voire inexistant (crêtes), et l'autre avec de moindres amplitudes thermiques, notamment en hiver à cause de la présence d'un manteau neigeux plus persistant (combes). Cette différence d'enneigement est également associée à une différence des communautés végétales dominant ces habitats. L'étude des communautés microbiennes de ces deux habitats a en grande partie fait l'objet d'une collaboration avec Philippe Choler et Florence Baptist (LECA), dont l'étude portait sur le comportement des flux de carbones dans ces milieux. La compréhension du rôle de la microflore des sols de ces deux habitats dans le recyclage de la biomasse végétale et du pool de matière organique locaux est en effet nécessaire pour prédire le devenir de ces stocks.

Nous nous sommes donc employés à caractériser les **dynamiques saisonnières des communautés bactériennes et fongiques de crêtes et de combes** sur deux années en utilisant la CE-SSCP couplée à une approche clonage/séquençage. Ces travaux sont valorisés par une publication de *ISME Journal* (Zinger et al., in press; cf. Chaptire II). Une caractérisation plus fine du comportement des communautés fongiques a été possible par la production de deux jeux de données de séquences issus de deux marqueurs moléculaires (ITS1 et région du gène de l'ARN ribosomal 28S). Cette étude a également consisté à développer une méthodologie d'analyse de jeux de données de séquences adaptée aux questions de structure et de diversité des communautés microbiennes par approches de « DNA barcoding », approche consistant à identifier la composition taxonomique des communautés en utilisant une région d'ADN standard. Ce travail fait l'objet d'une publication soumise à *Applied and Environmental Microbiology* (Zinger et al., submitted; cf. Chapitre II). Une étude de la structure phylogénétique des communautés bactériennes en réponse à ces habitats et à leur dynamique saisonnière fait également l'objet d'une publication soumise à *Environmental Microbiology* (Shanhavaz et al., submitted; Annexe B).

Enfin, la problématique de la dégradation de la matière organique de ces sols fait l'objet d'une collaboration transversale, avec plusieurs équipes du LECA (MicroAlp). Cette étude consiste à tester en conditions contrôlées l'impact de la température et de la qualité de la matière organique sur les processus de décomposition et sur la structure des communautés de

bactéries, champignons et crenarcheaotes. Ces travaux sont exposés dans une publication de *Environmental Microbiology* (Baptist et al., 2008; cf. Annexe C).

Les résultats prometteurs obtenus sur les travaux « crêtes et combes » nous ont encouragés à étendre la **caractérisation des communautés microbiennes alpines à l'échelle du paysage**. En effet, les écosystèmes alpins présentent une forte fragmentation et constituent une véritable mosaïque d'habitats. Les différentes communautés végétales des écosystèmes alpins présentent des différences de composition floristique, de diversité et de qualité de litière susceptibles d'influencer les communautés microbiennes. Nous avons donc étudié les communautés bactériennes, fongiques et crenarchaeotes à l'échelle du bassin versant, sous différentes communautés végétales en utilisant la CE-SSCP. Cette étude a été menée dans l'optique d'établir s'il existait un lien entre la distribution des communautés végétales et celle des communautés microbiennes. Ces travaux font l'objet d'une publication en préparation (Zinger L., Bousaria A., Baptist F., Geremia R.A, Choler P. *in prep.* ; Chapitre III)

Introduction: éléments de microbiologie environnementale

I. De l'enjeu de la microbiologie environnementale

1. Définition et bref historique

La microbiologie (du grec μῑκρος-, petit, -βίος-, vie, λογία, logique) a pour objet d'étude les micro-organismes. C'est une science relativement jeune en raison de la taille microscopique des organismes concernés. En effet, la première observation des micro-organismes n'a été rapportée qu'en 1677 par van Leeuwenhoek, grâce à ses optimisations du microscope. Durant la seconde moitié du XIXème siècle, Louis Pasteur met en place la culture de souches microbiennes, mettant fin au débat de la génération spontanée, et démontre l'implication de ces organismes dans la fermentation et les maladies. Parallèlement, Robert Koch formule ses postulats selon lesquels outre la responsabilité des micro-organismes dans les maladies, tous ne sont pas cultivables ni nécessairement pathogènes. Durant cet âge d'or de la microbiologie, Sergei Winogradsky et Martinus Beijerinck mettent en évidence le rôle des micro-organismes dans les cycles biogéochimiques (*e.g.* fixation de l'azote atmosphérique, dégradation de la cellulose, etc…). Dans les années 1930, Baas-Becking applique à ces organismes une approche écologique (Quispel, 1998). Depuis, les connaissances en microbiologie environnementale ne cessent de s'accroître par le biais d'avancées technologiques, qui connaissent un essor depuis le milieu du XXème siècle. (Prescott et al., 2003).

La microbiologie possède donc un pan environnemental englobant la microbiologie environnementale et l'écologie microbienne. Ces deux sous-disciplines se distinguent par l'échelle d'étude. Alors que l'écologie microbienne s'intéresse à l'étude du comportement et des activités des micro-organismes dans **leur environnement naturel immédiat**, la microbiologie environnementale vise à étudier les processus microbiens à **plus grande échelle** (Prescott et al., 2003). Comme nous pourrons le voir au cours de cette introduction, la microbiologie environnementale est une discipline vaste dont l'objectif est d'**étudier la**

réponse, l'action et la dynamique des communautés microbiennes sur/face aux changements environnementaux. Cette sous-discipline fait donc intervenir un large spectre de sciences du vivant comme la biologie moléculaire, l'écologie microbienne, la biogéochimie, etc...

2. Les micro-organismes, une source de diversité génétique

Les micro-organismes sont, par définition, des êtres microscopiques, pour la plupart unicellulaires. La majeure partie de ces organismes appartient aux règnes procaryotes Bactéries et Archaea, mais sont aussi représentés chez les eucaryotes comme les Protozoaires et les Fungi (Fig. 1). Les virus, bien que non cellulaires, sont également classés parmi les micro-organismes. L'utilisation de la phylogénétique a permis de mettre en évidence l'immense part que prennent les micro-organismes dans la diversité génétique du vivant par rapport aux macro-organismes (Fig. 1; Pace, 1997; Woese, 1998; Finlay, 2002).

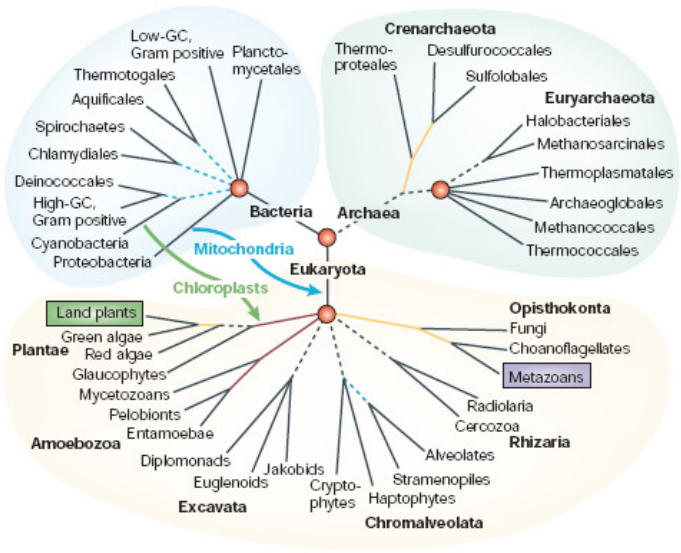


Figure 1 : Arbre phylogénétiques des êtres vivants. Il est plus simple d'indiquer ici les taxons ne faisant pas partie des micro-organismes : les plantes vertes (encadré vert), certaines algues, et de nombreux métazoaires (encadré violet). *Source : Delsuc et al. (2005).*

Les facteurs responsables d'une telle diversité sont multiples. En effet, ces organismes ont un long passé évolutif et un temps de génération relativement court. Les événements de mutation ou de recombinaison dans les génomes microbiens sont donc fréquents et s'accumulent depuis longtemps. A cela s'ajoutent les phénomènes de transferts horizontaux (échanges de gènes entre taxons différents) entre procaryotes (Ochman et al., 2000), de procaryotes à eucaryotes (Katz, 2002) mais aussi entre eucaryotes (Xie et al., 2008).

Tous ces remaniements génétiques confèrent aux micro-organismes un taux d'adaptation élevé. Parallèlement, la planète comporte une multitude de niches écologiques (cf . §III Encadré 2) dont le nombre explose si l'on se place à l'échelle microbienne. La diversité des microbes découle donc aussi d'une radiation adaptative dans des niches écologiques extrêmement diversifiées (revue dans Cohan and Koeppel, 2008), y compris dans les milieux extrêmes, inaccessibles aux organismes supérieurs (revu par Rothschild and Mancinelli, 2001).

La diversité microbienne est donc vaste. Par exemple, le nombre d'espèces bactériennes du sol a été estimé à plusieurs millions (Curtis et al., 2002), et à près d'un million chez les champignons (Bridge and Spooner, 2001; Hawksworth, 2001). De plus, les cellules procaryotes à elles seules représentent près de la moitié de la biomasse de la planète (Whitman et al., 1998). Ainsi, bien qu'ils soient invisibles à l'œil nu, les micro-organismes constituent une **composante essentielle du vivant** en termes d'abondance et de diversité.

3. Les micro-organismes, acteurs des processus environnementaux

La diversité génétique et la capacité des micro-organismes à coloniser des environnements divers, voire extrêmes, indiquent qu'ils possèdent des voies métaboliques variées (Pace, 1997). Cette propriété en fait des **acteurs clés** dans le fonctionnement des écosystèmes (Fig. 2). En effet, les micro-organismes interviennent largement dans les cycles biogéochimiques via la dégradation/minéralisation des déchets végétaux ou animaux (Hattenschwiler et al., 2005), et la réallocation des nutriments vers d'autres organismes. De ce fait et parce que les populations microbiennes constituent une biomasse importante et rapidement renouvelée, les micro-organismes occupent une place centrale dans les réseaux trophiques.

En agissant sur la qualité et/ou la disponibilité des ressources, les communautés microbiennes influencent la diversité et la fitness des espèces environnantes. Cet effet se traduit également à travers le type d'interactions que les membres de ces communautés entretiennent avec les autres organismes le long du continuum parasitisme-mutualisme (van der Heijden et al., 2008). Par exemple, les associations mycorhiziennes peuvent augmenter de façon plus ou moins directe la productivité des plantes (revue dans Johnson et al., 1997). A l'inverse, une espèce microbienne invasive peut avoir des conséquences dramatiques sur la diversité en espèce et le fonctionnement d'un écosystème (revue dans Desprez-Loustau et al., 2007). Ces interactions n'ont donc pas seulement un impact sur le fonctionnement des

écosystèmes, elles sont en plus de véritables moteurs de l'évolution par des effets de pression sélective ou de facilitation.

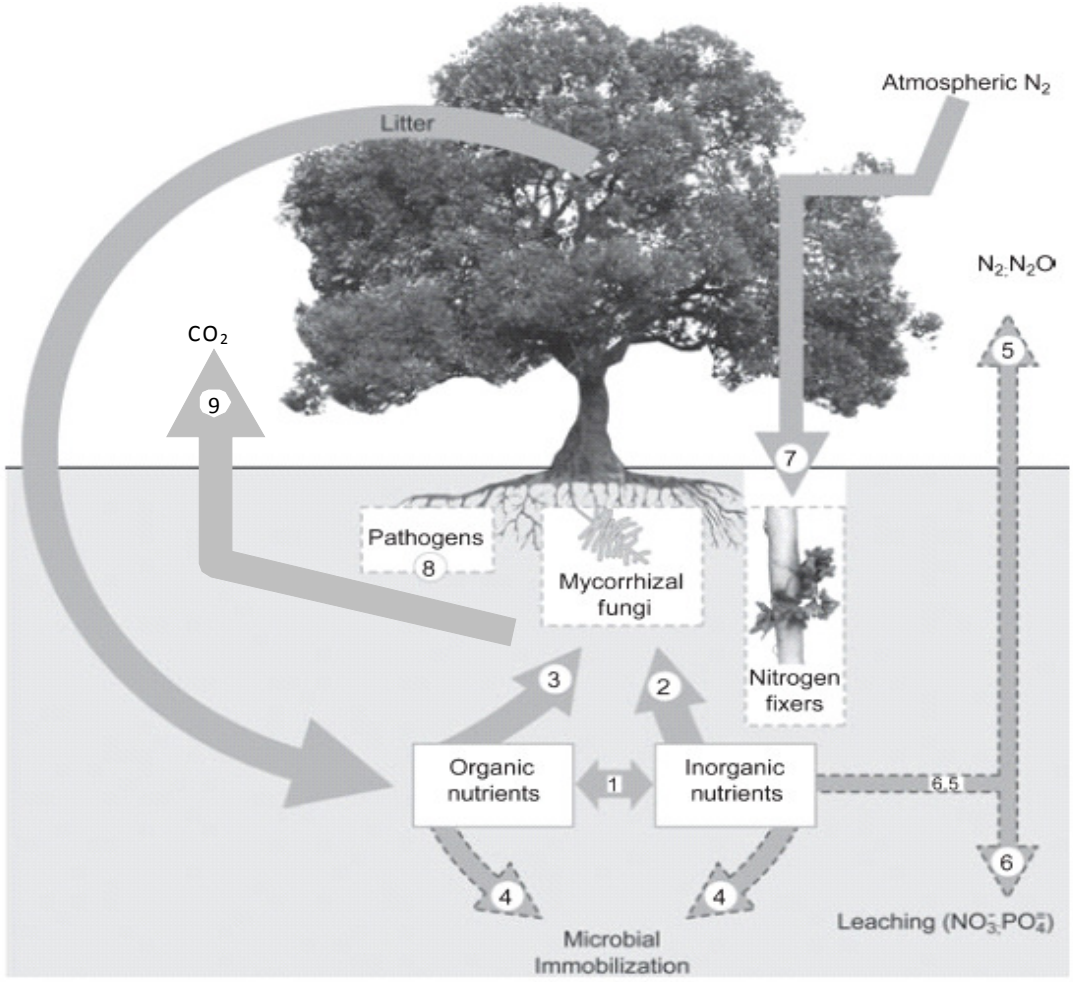


Figure 2 : Représentation schématique du rôle du compartiment microbien du sol. Les microbes agissent sur la disponibilité et la réallocation des ressources de façon positive par décomposition, transformation, et transport de la matière organique et des nutriments vers les plantes (1, 2 et 3), ou négative par séquestration des ressources dans leur biomasse ou dans la matière organique récalcitrante (4). La diminution de ressources est également due à la transformation de l'azote organique en des composés volatiles ou facilement lessivables (5, 6) qui peut néanmoins être acquis via les bactéries fixatrices d'azote atmosphérique (7). Les agents pathogènes induisent quant à eux une diminution de la productivité des plantes (8). Ces processus sont accompagnés d'efflux de CO_2 via la respiration des micro-organismes (9). *D'après van der Heijden et al.(2008)*

4. Les micro-organismes, dimension socio-économique

Les micro-organismes font partie de notre quotidien de par leur ubiquité et leur richesse. Responsables de nombreuses maladies aussi bien chez l'homme que chez d'autres espèces, ils constituent un réel problème sanitaire. Certaines périodes sombres de l'histoire résultent de l'activité pathogène particulièrement virulente de certains micro-organismes. Ainsi, l'invasion du champignon phytopathogène *Phytophthora infestans* qui frappa l'Irlande au milieu du XIXème siècle fit chuter la production de la pomme de terre, l'une des causes de la tristement célèbre « grande famine ». Citons également l'épidémie de peste noire, causée par la bactérie *Yersinia pestis*, qui décima la moitié de la population Européenne durant le XIVème siècle.

Par ce biais les microbes ont mauvaise presse. Pourtant, les relations microbes-hommes ne sont pas forcément négatives. Certains champignons produisent des fructifications consommables, la plus illustre étant la truffe. Les applications industrielles de leurs activités métaboliques sont vastes. L'industrie de l'agro-alimentaire en est un exemple frappant : de nombreuses souches microbiennes sont utilisées dans des processus de fermentation. Ils sont également largement utilisés dans l'industrie pharmaceutique pour la production d'antibiotiques ou autres médicaments. Capables de dégrader un large panel de molécules complexes, leurs enzymes sont utilisées en chimie de synthèse comme catalyseurs de réactions, et comme produits de nettoyage domestiques. Ces aptitudes enzymatiques en font des candidats précieux pour la bioremédiation (Dojka et al., 1998; Rothschild and Mancinelli, 2001).

Les micro-organismes occupent donc une place centrale dans le vivant par leur abondance, leur diversité, et leur implication dans les processus environnementaux. L'appréhension du compartiment microbien est donc d'une importance capitale pour une meilleure compréhension de ces processus, notamment dans un contexte de diminution de la biodiversité, de pollutions chroniques et, de réchauffement global.

II. Appréhension des communautés microbiennes, les outils

1. Les caractéristiques mesurables dans une communauté microbienne

L'étude d'une communauté microbienne peut se faire à travers différents indicateurs. Une communauté microbienne se définit par (i) sa biomasse, (ii) sa richesse, sa diversité, sa structure et sa composition en espèces, (iii) ses fonctions dans l'écosystème et la diversité de ces fonctions. La biomasse microbienne est responsable de nombreux processus biogéochimiques et constitue un réel réservoir de matière organique. Elle est sensible aux changements environnementaux tels que le pH, la couverture végétale ou la fertilité du milieu, ce qui en fait un bon indicateur de la qualité biologique et du fonctionnement des écosystèmes. De même, la composition et la diversité des communautés microbiennes sont fonction des conditions environnementales et peuvent renseigner sur l'état et le fonctionnement de l'écosystème. Enfin, les micro-organismes agissent sur l'environnement à travers un panel diversifié de voies métaboliques dont la caractérisation peut donner des indications sur les processus environnementaux qui s'y déroulent (Hurst, 2002, cf §III et §IV). Par exemple, des variations de biomasse, de structure et des activités métaboliques des communautés microbiennes ont été mises en évidence suite à l'augmentation de la quantité de métaux lourds (*e.g.* Cu, Zn, As ou Cd) (Pennanen et al., 1996; Yao et al., 2003; Lorenz et al., 2006).

Toutes ces caractéristiques peuvent être appréhendées via des techniques dites « traditionnelles », basées sur des réactions biochimiques et/ou sur la culture microbiologique, et des techniques moléculaires, plus récentes, basées sur l'ADN ou l'ARN (Table 1). Comme nous le verrons plus tard, cette thèse s'articule surtout autour de la structure et de la diversité des communautés microbiennes. Nous aborderons donc les aspects de biomasse et de fonction uniquement dans cette partie.

2. De l'importance de l'échantillonnage

L'échantillonnage joue un rôle crucial dans la caractérisation des communautés microbiennes. En effet, (i) les micro-organismes sont extrêmement diversifiés, (ii) il existe une relation aire/espèce chez ces organismes (cf. §III.2, revue dans Green and Bohannan, 2006) (iii) leur répartition spatiale varie à l'échelle microscopique. Alors que le point (i) est

inhérent à la communauté microbienne considérée et que sa précision relève plutôt des protocoles expérimentaux utilisés (*e.g.* profondeur de séquençage, cf §II.4), les points (ii) et (iii) sont déterminés par la stratégie d'échantillonnage.

La richesse des micro-organismes dépend effectivement de l'aire d'étude. En conséquence, le nombre de prélèvements doit être représentatif de la surface de l'écosystème étudié. Or, dans le cadre d'une étude comparative de deux sites à priori contrastés, la variabilité intra-site des communautés microbiennes peut atténuer l'effet inter-site. Cette limitation peut être contournée par l'analyse d'échantillons composites, c'est-à-dire par mélange des extraits d'ADN issus des réplicas spatiaux d'un même site (Schwarzenbach et al., 2007). En outre, la variabilité spatiale des espèces microbiennes est telle que la quantité de sol ou d'eau prélevés a un impact significatif sur les profils moléculaires (Ranjard et al., 2003; Venter et al., 2004). Ainsi, la stratégie d'échantillonnage est déterminante dans la caractérisation des communautés microbiennes et dans l'estimation de leur diversité.

3. Les méthodes dites « classiques »

La biomasse microbienne peut être estimée par comptage des cellules microbiennes ou par quantification de la part de carbone que représentent ces cellules (Table 1). D'autres méthodes permettent d'estimer cette biomasse, comme par exemple la spectroscopie proche infrarouge (NIRS) capable de détecter des composés carbonés typiquement microbiens (Cécillon et al., 2008). Certaines techniques moléculaires donnent également accès à ce type de mesure, comme l'hybridation fluorescente *in situ* (FISH), qui permet d'observer les micro-organismes *in situ* à l'aide de sondes fluorescentes, ou la PCR quantitative, qui donne accès à la quantité de copies d'un gène donné dont on peut tirer un nombre de cellules (Brouwer et al., 2003).

La composition spécifique des communautés microbiennes a longtemps été abordée par isolement de souches sur des milieux cultures plus ou moins sélectifs (Table 1). L'identification des souches isolées repose alors sur des critères morphologiques (mode de groupement des colonies/hyphes, pigmentation, forme des cellules ou des organes de fructification, etc…) et/ou par microscopie, complétés au besoin par des réactions biochimiques (réaction de gram, test de l'oxydase, etc…). Cependant, ces méthodes de culture ne sont pas forcément représentatives des communautés microbiennes étudiées : il est maintenant largement reconnu que plus de 99% des microbes n'ont pas encore été cultivés (revu par Amann et al., 1995).

Table 1 : Liste des techniques d'étude des communautés microbiennes, leurs applications, leurs avantages et inconvénients. *D'après Tiedje et al, (1999); Hurst (2002); Kirk et al. (2004).*
MPN : Most Probable Number ; DAPI : Di Aminido Phenyl lndol,; PLFA: PhosphoLipid Fatty Acids; CLPP: Communitiy-Level Physiological Profile; SIGR: Substrate Induced Growth Response ; ARDRA: Amplified Ribosomal DNA Restriction Analysis ; ARISA : Automated rRNA Intergenic Spacer Analysis ; T-RFLP : Terminal-Restriction Fragment Length Polymorphism ; T/DGGE : Temperature/Denaturing Gradient Gel Electrophoresis ; SSCP : Single Strand Conformation Polymorphism.

	Type de Méthode	Exemples	Informations	Avantages	Inconvénients
METHODES TRADITIONNELLES	Culture	MPN, Dénombrement sur boîte	Biomasse	Peu couteux, Rapide si le nombre d'échantillon est faible	Méthode lourde pour des études à grande échelle Biais des non-cultivables Ne reflète pas les conditions *in situ* Pas de renseignement sur les populations actives
		Isolement, criblage de souches	Identification morphologique et métabolique		
	Microscopie	Coloration de Gramm, DAPI	Identification de groupes Biomasse		Méthode lourde pour des études à grande échelle Pas de renseignement sur les populations actives
	Biochimie	Extraction-fumigation	Biomasse		
		PLFA	Biomasse Structure des communautés	Pas de biais des non-cultivables Accès au ratio de biomasse champignons/bactéries	Peu résolutif, surtout pour les champignons Pas de renseignement sur les populations actives
		CLPP, SIGR	Biomasse Potentiel métabolique	Peu coûteux et rapide Pas de biais des non-cultivables Suivi des populations actives	Pas d'identification des populations actives Ne reflète pas les conditions in situ
METHODES MOLECULAIRES	Approches basées sur la PCR (utilisation de marqueurs moléculaires)	DNA fingerprint: ARDRA, ARISA, T-RFLP, DGGE, TGGE, SSCP	Structure des communautés, diversité/richesse (méthodes semi-quantitatives)	Rapide Screening d'un grand nombre d'échantillons Pas de biais des non-cultivables Reproductible	Biais d'extraction et de PCR Choix d'amorces universelles Saturation de l'information pour des communautés complexes Pas/peu de renseignements taxonomiques
		Clonage/séquençage, pyroséquençage	Caractérisation taxonomique des communautés, diversité et richesse (méthodes semi-quantitatives)	Rapide Pas de biais des non-cultivables Reproductibles	Biais d'extraction et de PCR Choix d'amorces universelles Difficulté du traitement des grands jeux de données
	Méta-génomique et Méta-transcriptomique	Clonage/séquençage, pyroséquençage	Caractérisation taxonomique et métaboliques des communautés, diversité et richesse (méthodes quantitatives)	Rapide Pas de biais des non-cultivables Pas de biais de PCR	Biais d'extraction Difficulté du traitement des jeux de données

4. Les méthodes moléculaires

Dans les années 60, l'avènement de la biologie moléculaire a permis l'émergence d'un large éventail de méthodes basées sur l'ADN qui ont rapidement été appliquées à l'écologie des communautés chez les micro-organismes. A partir d'un ADN total extrait d'un échantillon, les communautés microbiennes sont appréhendées soit (i) dans leur ensemble, par des approches de méta-génomique, soit (ii) de manière dirigée, par amplification par PCR de certaines régions du génome communes à l'ensemble des organismes étudiés (Table 1). Brièvement, un marqueur moléculaire est une région d'ADN variable, permettant la distinction d'un maximum de taxons, flanquée de régions suffisamment conservées, permettant l'amplification de l'ADN d'un maximum de taxons. Ce sont principalement les gènes ribosomaux qui sont utilisés pour l'étude des micro-organismes (*e.g*. gènes de l'ARNr 16S chez les procaryotes, ARNr 18S ou 28S et ITS chez les eucaryotes). Toutes ces méthodes sont cependant soumises au même biais : l'extraction de l'ADN n'est pas exhaustive (Frostegard et al., 1999; Martin-Laurent et al., 2001).

Actuellement, ce sont les méthodes basées sur la PCR, et en particulier les méthodes de type « DNA fingerprint » (empreinte moléculaire) les plus couramment utilisées parce qu'elles permettent le suivi de nombreux échantillons en demeurant accessibles en termes de temps et de coûts. Ces méthodes, reposant sur l'électrophorèse, permettent de séparer des fragments d'ADN selon leur taille (ARISA, ARDRA, T-RFLP) et leur composition en bases (DGGE, TGGE, SSCP). Toutes reposent sur l'amplification par PCR de fragments d'ADN à partir d'un extrait en raison de (i) une quantité d'ADN inférieure aux seuils de détection et (ii) la complexité de l'extrait d'ADN, constitué d'un ensemble de génomes de nombreux individus appartenant à des taxons divers. Les techniques d'empreintes moléculaires se distinguent aussi par le type de marqueurs moléculaires employé (ARDRA, ARISA) ou l'utilisation d'enzymes de restrictions (ARDRA, T-RFLP). Elles donnent cependant accès au même type d'information, à savoir un profil moléculaire où le nombre et l'intensité des pics reflètent la richesse et l'abondance des phylotypes microbiens (cf. Chapitre I). Ces profils permettent donc de mettre en évidence des variations de structure et de diversité (Loisel et al., 2006) des communautés induites par des changements environnementaux. Certaines d'entre elles montrent un degré de précision supérieur aux autres (la T-RFLP notamment, Tiedje et al., 1999) mais nécessitent des manipulations supplémentaires, et sont donc plus difficilement

applicables dans le cadre d'études à grande échelle. Cette discussion fait, en partie, l'objet du Chapitre I du présent manuscrit.

Encadré 1 : Les biais liés au marqueur moléculaire et à la PCR

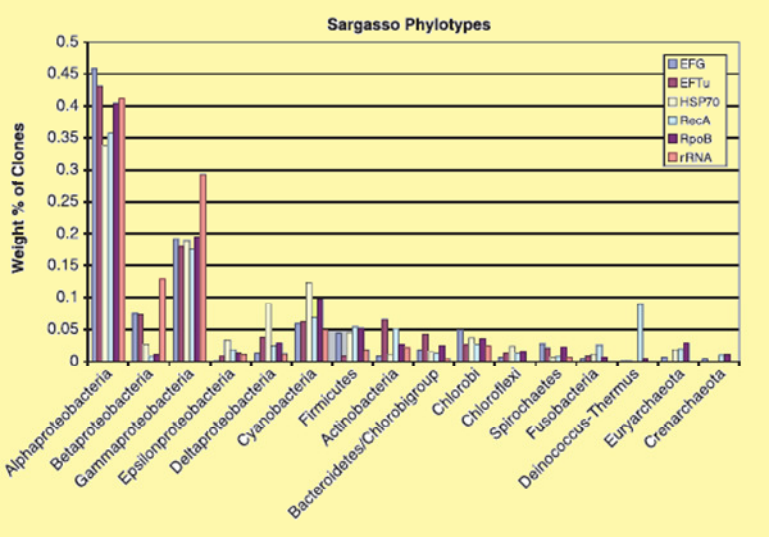
1/ Le choix du marqueur moléculaire est déterminant dans la finesse et la justesse de l'analyse. En effet, tous les marqueurs ne présentent pas le même degré d'universalité. Il est également possible qu'un marqueur ne soit pas résolutif pour tous les taxons ciblés. Enfin, les amorces utilisées pour ce marqueur peuvent montrer une certaine spécificité (Anderson et al., 2003). De plus, certains gènes, notamment les gènes ribosomaux, sont répétés dans le génome et le nombre de répétition diffère d'un taxon à l'autre (Fig. E1). L'abondance des séquences obtenues n'est donc pas forcément représentative de la communauté réelle.

2/L'ADN polymérase provoque des erreurs pendant l'amplification par PCR. Les plus mineurs sont les erreurs de « recopiage » qui peuvent influencer les résultats (Qiu et al., 2001). Il existe d'autres enzymes plus fidèles de part leur activité exonucléasique (proof-reading polymerases). L'ADN polymérase ajoute une ou plusieurs bases d'adénine en fin de synthèse du brin d'ADN (Brownstein et al., 1996). Ce phénomène modifie la taille initiale du fragment. Il est donc critique pour les analyses de type « DNA fingerprint ». Les biais liés à l'ADN polymérase sont développés dans l'Annexe A.

3/L'amplification de mélanges complexes donne lieu à des artéfacts. En effet, il existe des phénomènes d'amplification préférentielle pendant la PCR due aux caractéristiques des fragments d'ADN, qui ne sont pas prédictibles dans le cadre d'étude des communautés (Polz and Cavanaugh, 1998). De plus, les fragments d'un mélange complexe interagissent entre eux. Cela peut donner lieu à la formation d'hétéroduplexes, par événements de recombinaison artificielle, ou de chimères, par saut de l'ADN polymérase d'un fragment à l'autre (Qiu et al., 2001; Thompson et al., 2002). Ces événements sont difficilement évitables.

La PCR en émulsion permet de contourner ces artéfacts en amplifiant chaque fragment d'ADN isolément dans de microgouttelettes, évitant ainsi l'amplification préférentielle ou la formation de chimères (Nakano et al., 2003).

Figure E1 : Diversité bactérienne de la mer des Sargasses. Chaque couleur correspond à un marqueur moléculaire différent. Notons la différence d'information que génère chaque marqueur. Par exemple, les gènes ribosomaux surestiment l'abondance des Gammaprotéobactéries. *Source Venter et al. (2004).*



L'utilisation du clonage/séquençage de marqueurs moléculaires permet une analyse plus fine des communautés microbiennes. Brièvement, le séquençage d'un mélange de molécules d'ADN n'est pas possible. Cette limite est contournée par une étape de clonage, qui permet d'isoler chaque fragment d'ADN avant le séquençage. Cette approche est souvent employée de façon complémentaire avec les techniques de cultures pour préciser l'identification taxonomique d'une souche. Cependant, son application principale demeure l'étude de communautés microbiennes complexes car il donne accès non seulement à la composition, mais aussi à la richesse et la diversité des communautés microbiennes. Malheureusement, la diversité microbienne est si vaste qu'il est difficile de la caractériser pleinement sans le séquençage d'un grand nombre de clones. En effet, Quince et al. (2008) ont estimé à 4 millions le nombre de séquences de gène de l'ADNr 16S nécessaire pour décrire 90% d'un métagenome complexe. L'effort de séquençage peut être amélioré par l'utilisation des nouvelles techniques de séquençage massif, comme le pyroséquençage (Margulies et al., 2005). Il est cependant nécessaire de garder en tête que les techniques basées sur l'amplification par PCR sont soumises à des biais (Encadré 1), limitations auxquelles les approches méta-génomiques ne sont pas soumises puisqu'elles consistent à séquencer de façon non dirigée l'ensemble des génomes contenus dans un échantillon. L'émergence des nouvelles technologies de séquençage est une étape majeure dans la caractérisation des communautés microbiennes, puisqu'elle permet d'augmenter de façon significative l'effort de séquençage. Cependant ces techniques génèrent parallèlement une autre limite ; celle de l'analyse de jeux de données contenant des centaines de milliers de séquences (cf. chapitre 3).

Les méthodes d'analyse des communautés microbiennes sont donc variées, mais aucune n'est exhaustive. Chacune d'entre elles présente ses limites qui se répercutent de manière différente sur les résultats obtenus. Ce n'est donc que par la définition d'une bonne stratégie d'échantillonnage et par la conjugaison de techniques d'approches complémentaires adaptées à la question biologique que l'appréhension des communautés microbiennes peut être optimisée.

III. Appréhension des communautés microbiennes, les concepts

1. Comment définir une espèce microbienne ?

La compréhension du compartiment microbien et de son rôle dans l'environnement nécessite l'identification des individus qui le composent. Cette identification repose sur la classification de ces individus en groupes partageant un certains nombre de caractéristiques. Ces caractéristiques peuvent être génétiques, fonctionnelles, ou systématiques (*i.e.* taxonomiques). Or ces critères de classification possèdent des niveaux d'organisation hiérarchiques. Par exemple *:* doit-on grouper ensemble les individus partageant 70 ou 90% de leur génome ? Doit-on classer les individus selon leur capacité à dégrader certains composés ou selon le type de voies métaboliques employées dans la dégradation de ces composés ? Puisqu'une unité universelle de classification des organismes relève de l'utopie, le regroupement des individus selon certains critères nécessite la fixation de seuils. Bien que la taxonomie présente également une organisation hiérarchique, les seuils définis par celle-ci sont fixes, utilisés depuis des siècles, et paraissent donc plus intuitifs. Ainsi, la plupart des études en écologie des communautés sont basées sur une unité, l'espèce.

Chez les macro-organismes, une espèce se définit principalement par l'isolement reproductif des individus qui la composent. Au sein d'une population, cette spéciation peut être due soit à un isolement physique qui mène à la spéciation par dérive génétique (spéciation allopatrique), soit à des événements de recombinaison génétique limitant le croisement de certains individus (spéciation sympatrique). Or, cette notion d'espèce est beaucoup plus complexe chez les micro-organismes (Encadré 2). En effet, ils présentent la plupart du temps une reproduction clonale rendant difficile l'observation d'un isolement génétique franc (Fig. 3a ; Taylor et al., 2000; Acinas et al., 2004). La complexité de cette notion est accentuée par les phénomènes de transfert horizontaux, courant chez les micro-organismes (Ochman et al., 2000, cf. §I.2), qui tendent à générer des génomes « en mosaïque » (Tyson et al., 2004). Enfin, les micro-organismes possèdent une importante plasticité phénotypique au niveau « intra-spécifique », que ce soit en termes de morphologie (Rainey and Travisano, 1998, Fig. 3b) ou d'activité enzymatique (revue dans Deitsch et al., 1997; Buee et al., 2007) rendant impossible toute classification sur critères morphologiques et éco-physiologiques. Ainsi, la découverte d'espèces cryptiques au sein d'espèces microbiennes

prédéfinies a amené la communauté scientifique à revisiter le concept d'espèce chez les micro-organismes (Encadré 2)

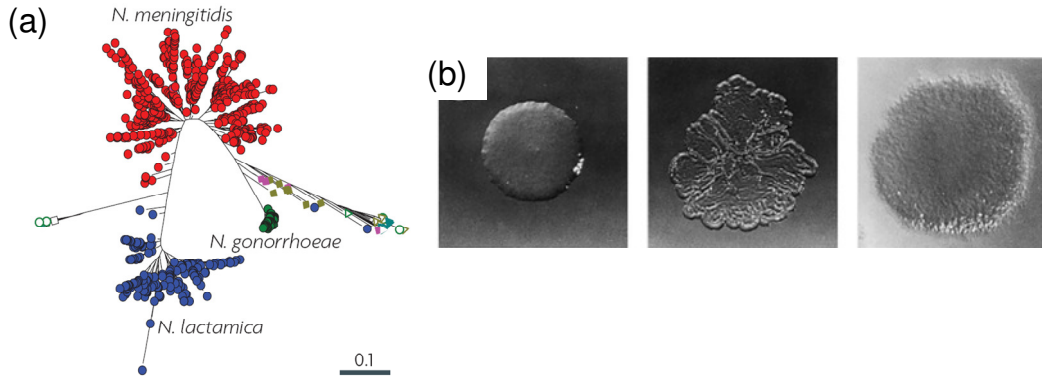


Figure 3 : Des difficultés dans la classification des micro-organismes. **(a)** Arbre Bayesien d'espèces bactériennes (une couleur par espèce) du genre *Nesseria*. Alors que les espèces rouges, bleues et vertes paraissent clairement définies, les autres ne présentent pas d'isolement génétique franc. *Source : (Achtman and Wagner, 2008).* **(b)** diversité morphologique des colonies de *Pseudomonas fluorescens* SBW25 en réponse à des variations environnementales. *Source : Rainey and Travisano (1998).*

Dans ce contexte, il est ardu d'établir des groupes d'individus distincts dans une communauté microbienne. De plus, les différents concepts de l'espèce microbienne (Encadré 2) sont difficilement applicables à l'échelle de la communauté parce qu'ils nécessitent l'acquisition et la comparaison de génomes entiers (cf. §II.4). Malgré ces limitations, le comportement des communautés microbienne et de leur diversité sont abondamment documentés (*e.g.* Venter et al., 2004; O'Brien et al., 2005; Fierer and Jackson, 2006; Fierer et al., 2007b). Ces études sont basées sur la **systématique moléculaire** soit (i) par une approche multilocus (screening et phylogénie de plusieurs gènes), qui reste difficilement applicable à l'échelle de la communauté, soit (ii) par criblage et/ou phylogénie des gènes ribosomaux, consistant à étudier l'identité, la richesse, la diversité et/ou l'affiliation phylogénétique des unités taxonomiques opérationnelles (OTUs), composées par des rybotypes (séquences ribosomales) similaires. Bien que cette approche ne présente pas de justification théorique (Encadré 2) et soit limitée par les techniques expérimentales (cf. § II.4), elle a néanmoins permis de s'affranchir des limites causées par le manque de consensus autour du concept d'espèce microbienne, et par notre incapacité actuelle à isoler en culture la majorité de ces organismes (revu par Amann et al., 1995). Cette approche a en outre donné accès à certains

phénomènes dans le comportement des communautés microbiennes que nous évoquerons plus tard.

Encadré 2 : les différents concepts d'espèce microbienne

Les critères de classification des micro-organismes en espèces sont maintenant divers. Certains considèrent qu'une espèce doit être monophylétique et présenter une cohésion génomique et phénotypique. D'autre pensent qu'elle se définit en fonction du taux de transferts horizontaux, plus élevé au sein d'un groupe qu'entre groupes Certains considèrent enfin que le concept d'espèce n'est pas applicable aux microbes (revu par Achtman and Wagner, 2008).

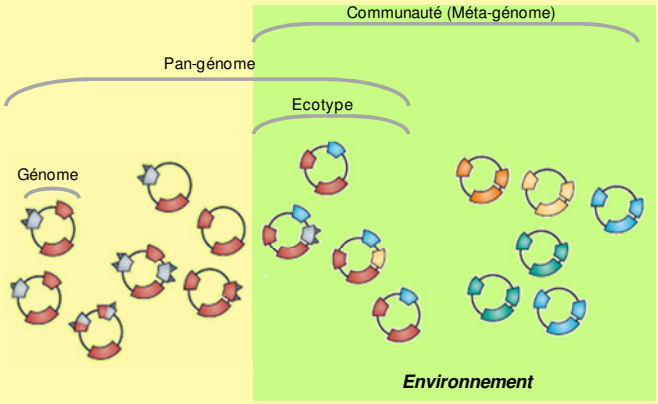
Malgré ce manque de consensus, il apparaît que les microbes peuvent être classifiés par le concept de **pan-génome**. Celui-ci comprend un pool de gènes commun à tous les individus, le « core-genome », et un répertoire de gènes peu/pas partagés, le « dispensable genome », responsable de la plasticité écophysiologique du groupe et lui conférant son pouvoir adaptatif (Medini et al., 2008, Fig. E2).

La notion de pan-génome englobe celle d'**écotype,** qui réfère à un groupe d'individus écologiquement similaires à un autre mais présentant une certaine cohésion génétique causée par la sélection et/ou la dérive génétique (revu par Cohan and Perry, 2007, Fig. E2).

La définition d'espèce chez les micro-eucaryotes semble suivre le même schéma. Cependant, les concepts d'espèce chez ces organismes sont plutôt d'ordre opérationnel (*i.e.* méthodologiques, Taylor et al., 2000) que théorique (mais voir Kohn, 2005; Giraud et al., 2008)

Le concept d'espèce microbienne apparait donc comme une sorte de **continuum** où certains individus forment des groupes distincts et d'autres des groupes moins définis (revue dans Achtman and Wagner, 2008), Fig. 3a). Les critères de classification des microbes dépendent donc de la question biologique posée.

Figure E2 : **Représentation schématique des principaux concepts d'espèce microbienne**. *D'après Medini et al. (2008)*



2. Les patrons de distribution spatiale des micro-organismes

Nous avons précédemment vu que les micro-organismes ont colonisé la quasi-totalité de la planète. Mais malgré cette ubiquité, les « espèces » microbiennes et leur assemblage suivent-ils des patrons de distribution similaires à ceux des macro-organismes (Encadré 3) ?

S'il on en croit le vieil adage de la microbiologie défini par Baas-Becking et inspiré des travaux de Beijerinck, « *everything is everywhere*, but, *the environment selects* » (Quispel, 1998). Ceci implique que les micro-organismes ont une biomasse et une capacité de dispersion telles que leur présence est possible dans n'importe quel environnement, mais que les contraintes environnementales locales ne favorisent que certaines espèces. Cette doctrine est toujours débattue à ce jour, certains supportant le cosmopolitisme des micro-organismes (Finlay, 2002; Fenchel and Finlay, 2003), et d'autres soutenant le contraire (*e.g.* Cho and Tiedje, 2000; Whitaker et al., 2003; Taylor et al., 2006; Halling et al., 2008).

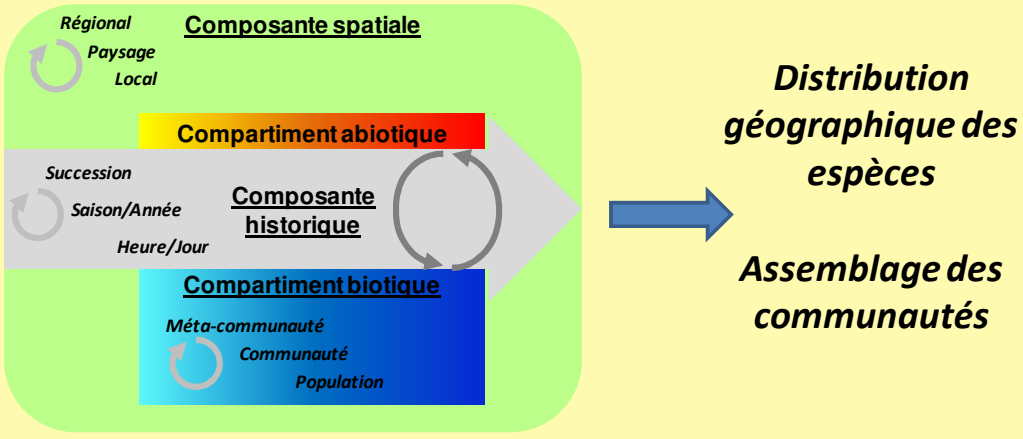
Encadré 3 : Quelques notions d'écologie des communautés

La distribution des organismes découle de différents facteurs. Premièrement, les **facteurs écologiques** (*e.g.* température, disponibilité en eau, interactions biotiques, etc…) agissent comme des filtres sur les espèces (Belyea and Lancaster, 1999; Lavorel and Garnier, 2002, Fig. E3). En effet, les conditions environnementales définissent des niches écologiques. Ces niches présentent différents niveaux d'organisation, *e.g.* la caractéristique « fertilité » englobe les caractéristiques de quantités de nitrates et de phosphore. A l'intérieur de ces niches, deux espèces ne peuvent coexister durablement que si leurs besoins diffèrent à un niveau d'organisation inférieur de la niche (principe d'exclusion compétitive). L'assemblage des espèces sur un site donné résulte donc en partie de l'assemblage des niches de ce site.

Deuxièmement, les demandes et les réponses d'une espèce a vis-à-vis d'une niche la rendent plus ou moins apte à s'adapter et à se maintenir dans un milieu. Ces **facteurs biologiques**, inhérents à l'espèce, sont de même ordre chez les espèces occupant des niches similaires, même si celles-ci sont géographiquement distantes. Les taux de dispersion et de spéciation de l'espèce, qui déterminent sa capacité à coloniser le milieu, sont également des facteurs biologiques.

Enfin, les interactions biotique/abiotique sont dynamiques (Fig. E3). La distribution des espèces dépend donc aussi de **facteurs historiques**, qui renvoient à la succession de peuplements et de conditions environnementales qui ont contribué à l'état actuel du site considéré (Bardgett, 2005; Hughes Martiny et al., 2006).

Figure E3 : Représentation schématique du fonctionnement d'un système. Il existe trois grandes composantes en biogéographie : la structure biologique considérée, l'espace, et le temps (soulignés), chacune présentant différents niveaux d'organisation. Ces composantes interagissent, et leurs différents niveaux d'organisation ont une répercussion les uns sur les autres



Avant d'aller plus loin dans ce débat, il est nécessaire de redéfinir certaines caractéristiques propres aux micro-organismes. Chez les macro-organismes, une communauté se structure selon un schéma bien établi. Elle est composée de quelques espèces largement dominantes, accompagnées par des subordonnées. On y trouve aussi des espèces dites de « transition », souvent insubordonnées aux espèces dominantes, plus rares, et beaucoup plus diverses (Grime, 1998) Les communautés microbiennes suivent des règles similaires (Hughes et al., 2001; Zhou et al., 2002; Tedersoo et al., 2008) mais montrent une diversité d'espèces rares bien supérieure à celle des macro-organismes, désignée comme la « biosphère rare » (Sogin et al., 2006). Tout comme chez les végétaux, la diversité de ces espèces rares pourrait être impliquée dans la résilience des écosystèmes suite à différents degrés de perturbations (Grime, 1998; Sogin et al., 2006). De la même manière, la **relation aire-espèce** existant chez les macro-organismes a également été rapportée chez les micro-organismes. Cette relation apparaît généralement plus faible en raison de leur diversité. Cependant, cette observation est soumise à caution étant donné (i) qu'aucune technique d'approche des communautés n'est exhaustive (cf. §II), (ii) que cette relation varie avec la résolution taxonomique, et (iii) que les études sur le sujet n'utilisent pas des stratégies d'échantillonnage et des techniques comparables (revue dans Green and Bohannan, 2006).

Les microbes montrent aussi certaines caractéristiques fondamentales impliquées dans leurs patrons de distribution. La plupart possèdent un **taux de dispersion élevé** de part leur petite taille ou celle de leurs spores. Cette dispersion se fait de manière passive, *via* l'atmosphère et l'eau, mais peut aussi être inféodée à la migration d'autres organismes. De plus, les micro-organismes ont un **taux de spéciation élevé** en raison (i) d'une action de la sélection rapide due à un temps de génération court, et (ii) de l'acquisition de traits écologiques avantageux par transferts horizontaux de gènes provenant des micro-organismes autochtones (cf §I.2 et §III.1). En raison de ces caractéristiques, il est possible que les microbes connaissent un **taux d'extinction plus faible**, sachant de plus que certains d'entre eux sont capables de sporuler ou d'entrer dans des phases de vie leur permettant de résister aux stress environnementaux (Horner-Devine et al., 2004; Kohn, 2005; Ramette and Tiedje, 2007).

Il semble que l'assemblage des communautés microbiennes soit soumis à des règles équivalentes à celles influençant les macro-organismes (Encadré 3). Notre incapacité à cultiver la majorité des micro-organismes indique déjà leur inégalité face aux conditions environnementales. De nombreuses études abondent dans ce sens et ont pu mettre en évidence

l'impact du pH, de la disponibilité des ressources ou de l'utilisation des terres sur la structure et la diversité des communautés microbiennes (*e.g.* Zhou et al., 2002; Green et al., 2004; Fierer and Jackson, 2006; Kasel et al., 2008; Lauber et al., 2008; cf §IV), donnant à penser que ces communautés suivent des patrons de **distributions non-aléatoires**, en fonction des conditions environnementales.

De plus, d'autres études ont également révélé une certaine dissimilarité des communautés microbiennes (Green et al., 2004; Fulthorpe et al., 2008), voire un certain degré d'endémisme des espèces, avec l'augmentation de la distance géographique (Cho and Tiedje, 2000; Taylor et al., 2006; Halling et al., 2008). Ces études montrent que l'isolement géographique, et par conséquent historique, est un moteur de spéciation qui influence la composition des communautés microbiennes (Fig. 4, revue dans Hughes Martiny et al., 2006), rejoignant par ce fait les facteurs régissant la distribution des macro-organismes (Encadré 3). En somme, les communautés microbiennes tendent à se ressembler si leur environnement se ressemble, quelle que soit l'échelle d'observation (*i.e.* province ou ensemble de provinces), mais tendent à se différencier avec l'éloignement géographique (Fig. 4, revu Hughes Martiny et al., 2006). Cependant, la majorité des études portant sur l'endémisme des micro-organismes en fonction de la distance géographique ont été menées dans des écosystèmes possédant des caractéristiques radicalement différentes (*e.g.* couvert végétal, type de sol, etc..). Dans ce contexte, il est difficile de dissocier la part des facteurs écologiques de celle de l'éloignement géographique dans la différenciation des communautés ou dans la spéciation. Par exemple, (Fierer et al., 2007a) ont observé un effet de l'environnement supérieur à celui de la distance géographique à l'échelle du bassin versant.

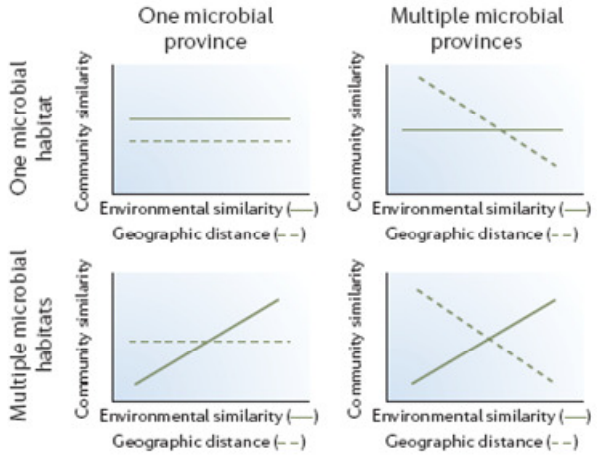


Figure 4 : Effets supposés des composantes écologiques et historiques sur les communautés microbiennes. Un habitat définit un type de conditions environnementales (conditions abiotiques et couvert végétal). Une province représente une aire géographique dont le passé évolutif est homogène (*e.g.* un continent). Une province inclut donc plusieurs habitats. *Source Hughes Martiny et al. (2006).*

En considérant toutes ces caractéristiques propres aux micro-organismes, une « espèce » microbienne est susceptible d'avoir une aire de répartition beaucoup plus étendue qu'une macro-espèce *via* un taux de dispersion élevé. Cette étendue apparaît néanmoins limitée par le fort taux de spéciation de ces organismes couplé à la distribution des niches écologiques, ainsi que par la dépendance de certains de ces organismes à un hôte. Cependant, un taux d'extinction faible autorise la présence d'espèces inadaptées à l'environnement par les phénomènes que nous avons précédemment cité. Cette propriété corrèle avec la diversité vaste de la « rare biosphère » observée par (Sogin et al., 2006) et composée de nombreuses espèces de très faible abondance. Cette biosphère apparaît comme un accumulateur, indépendant du succès de l'espèce, de spores/souches se trouvant ou s'étant trouvées dans le milieu, rappelant ainsi les « banques de graines » observées chez les végétaux. Or, une telle diversité d'espèces rares est difficilement accessible de manière exhaustive avec les techniques utilisées en microbiologie environnementale (cf §II), et la part des populations effectivement actives dans ces communautés l'est encore plus. La composition spécifique réelle de ces communautés reste donc inaccessible.

Ainsi, le débat d'une biogéographie des micro-organismes semble donc davantage un débat sémantique plutôt que conceptuel. La doctrine de Baas-Becking possède un sens large, aux interprétations variées (de Wit and Bouvier, 2006). Les acteurs de ce débat ne se contredisent pas quant à l'impact indéniable des facteurs environnementaux sur la structure des communautés microbiennes, mais n'utilisent ni les mêmes critères de définition de l'espèce, ni la même profondeur d'échantillonnage, ce qui conduit forcément à une divergence d'opinion face à la question de biogéographie microbienne.

3. Quid de la dynamique des communautés microbiennes ?

L'assemblage des communautés varie non seulement dans l'espace (Encadré 3), mais aussi dans le temps. En effet, les facteurs biotiques et abiotiques responsables de la présence d'une espèce sont eux-mêmes soumis à une dynamique qui s'opère sur une hiérarchie de pas de temps. Ainsi, les processus de colonisation et d'extinction d'espèces sur un site donné suite à une modification de l'habitat sont définis en écologie comme des **successions**. Cette succession suit une trajectoire dépendant de la nature de la perturbation de l'habitat, des espèces amenées à coloniser le milieu et/ou déjà présentes dans ce milieu sous forme latente, ainsi que des conditions climatiques. Ces processus s'opèrent de manière **continue**, sur des pas de temps de l'ordre de l'année ou de la décennie chez les macro-organismes.

A l'échelle microbienne, l'impact des changements environnementaux sur l'assemblage des communautés peuvent s'opérer sur des pas de temps beaucoup plus courts (journée, saisons). En effet, à un niveau local, la disponibilité des ressources peut varier à très court terme et ainsi rapidement influencer la structure des communautés microbiennes (Pinhassi et al., 1999; Bardgett et al., 2005b; Schmidt et al., 2007). A l'échelle de la semaine ou du mois, des stress tels que des événements de gel-dégel, de sécheresse/humidification, ou de pollution peuvent affecter la communauté (Bardgett, 2005). Ces changements s'opèrent également en fonction des saisons, avec le développement de la végétation et les fluctuations climatiques (Murray et al., 1998; Bardgett et al., 2005b; Nemergut et al., 2005; Schmidt et al., 2007). Enfin, l'évolution des communautés microbiennes varie sur des pas de temps beaucoup plus long, en parallèle à la succession des communautés végétales (Ohtonen et al., 1999; Bardgett et al., 2005b).

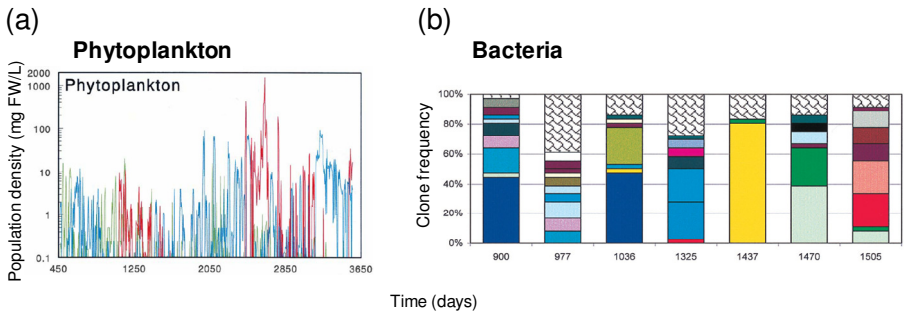


Figure 5 : Dynamique des communautés microbiennes dans des environnements stables. **(a)** Dynamique d'une communauté de phytoplancton d'une dizaine d'espèces (vert : flagellés verts, bleu : pico-phytoplancton procaryote, rouge : les diatoméées du genre *Melosira. Source : Scheffer et al. (2003).* **(b)/** Dynamique de la communauté bactérienne d'un réacteur méthanogénique (chaque couleur représente un OTU, le motif représente des séquences uniques). *Source : Fernandez et al. (1999)*

Il est également possible d'observer des fluctuations des communautés microbiennes indépendamment des conditions environnementales (hors disponibilité des ressources). Les interactions entre les protagonistes de la communauté sont de natures diverses et s'articulent le long du continuum parasitisme-mutualisme (cf. §IV.4). Enfin, ces communautés forment des chaînes trophiques, et transforment continuellement les ressources du milieu de manière qualitative et quantitative. Ainsi, plusieurs auteurs ont mis en évidence l'instabilité des populations microbiennes dans des conditions pourtant stables (Fernandez et al., 1999; Scheffer et al., 2003; Becks et al., 2005; Fig. 5). Ces fluctuations ont été jugées d'ordre

chaotique (Becks et al., 2005), mais ne semblent pas modifier le fonctionnement de l'écosystème (Fernandez et al., 1999).

La dynamique des communautés microbiennes semble donc liée à des mécanismes complexes, encore difficiles à cerner en raison des facteurs indénombrables impliqués dans ce processus. Néanmoins, ce comportement apparemment chaotique peut être expliqué par l'opportunisme écologique des espèces de la communauté en réponse à des changements infimes des conditions environnementales (Rainey et al., 2005).

En résumé, il existe un réel manque de cadre conceptuel dans l'appréhension du compartiment microbien. Les concepts tirés des patrons de diversité, de répartition et de dynamique des macro-organismes ne peuvent être appliqués directement aux micro-organismes en raison de leur petite taille, de leur grande diversité et d'une dynamique des communautés rapides. Finalement, la question n'est pas de savoir si les micro-organsimes suivent les mêmes lois que les macro-organismes, mais plutôt de savoir quelle est l'échelle à laquelle les patrons microbiens se rapprochent au mieux de ceux des macro-organismes (Green and Bohannan, 2006).

IV. Les communautés microbiennes du sol, une étape de plus vers la complexité

1. De l'importance des sols

En raison d'une diversité, d'une abondance et d'une dispersion élevées, les micro-organismes ont colonisé la quasi-totalité de la surface planétaire. De nombreuses études les décrivent dans des écosystèmes marins (*e.g.* (Moon-van der Staay et al., 2001; Thorseth et al., 2001; Venter et al., 2004)), des environnements extrêmes (*e.g.* milieux acides, hyper salins, anoxiques, secs, contaminés, etc…, revu par Rothschild and Mancinelli, 2001), des environnements biologiques (*e.g.* tube digestif, racines, etc; Backhed et al., 2005), mais surtout dans les sols (revu par Tiedje et al., 1999; Torsvik and Ovreas, 2002; Anderson and Cairney, 2004; Foissner, 2006). Par exemple, un gramme de sol provenant d'un champ peut contenir 10^9 bactéries, 10^5 protozoaires et 1 km de mycélium (Young and Ritz, 2005).

Le sol se définit comme étant la couche superficielle meuble de l'écorce terrestre. Il est issu d'interactions entre une matrice physico-chimique de composition extrêmement variable et une grande diversité d'organismes. Il n'est donc pas qu'un simple support des êtres vivants ; il constitue aussi un important stock de matière organique et minérale, abritant une grande diversité d'êtres vivants (Fig. 6). En conséquence, il s'y déroule de nombreux processus biogéochimiques dont les acteurs sont variés. Le sol est donc un véritable **carrefour multifonctionnel** (Gobat et al., 2003) et occupe une place centrale dans le fonctionnement des écosystèmes terrestres. Représentant près d'un tiers de la surface de la planète, les services rendus par le sol s'appliquent non seulement aux milieux naturels, mais aussi aux systèmes anthropiques en regard de l'agriculture ou la gestion des déchets (Costanza et al., 1997).

Le sol est un compartiment extrêmement **hétérogène**. Ses caractéristiques varient à petite échelle, selon la répartition des pores, des agrégats, des racines, des nutriments et de l'eau (Young and Crawford, 2004, Fig. 6), mais aussi à plus grande échelle, en fonction de la roche mère, du climat et de la couverture végétale (Hooper et al., 2000; Ettema and Wardle, 2002). De la même manière, les caractéristiques du sol peuvent changer dans l'heure ou dans la journée, d'une saison à l'autre, et évoluent au cours des successions (Bardgett et al., 2005b). C'est donc un compartiment diversifié et dynamique ; en 4 dimensions : surface,

profondeur et temps. De part sa complexité, il constitue une réelle « boîte noire » des sciences environnementales.

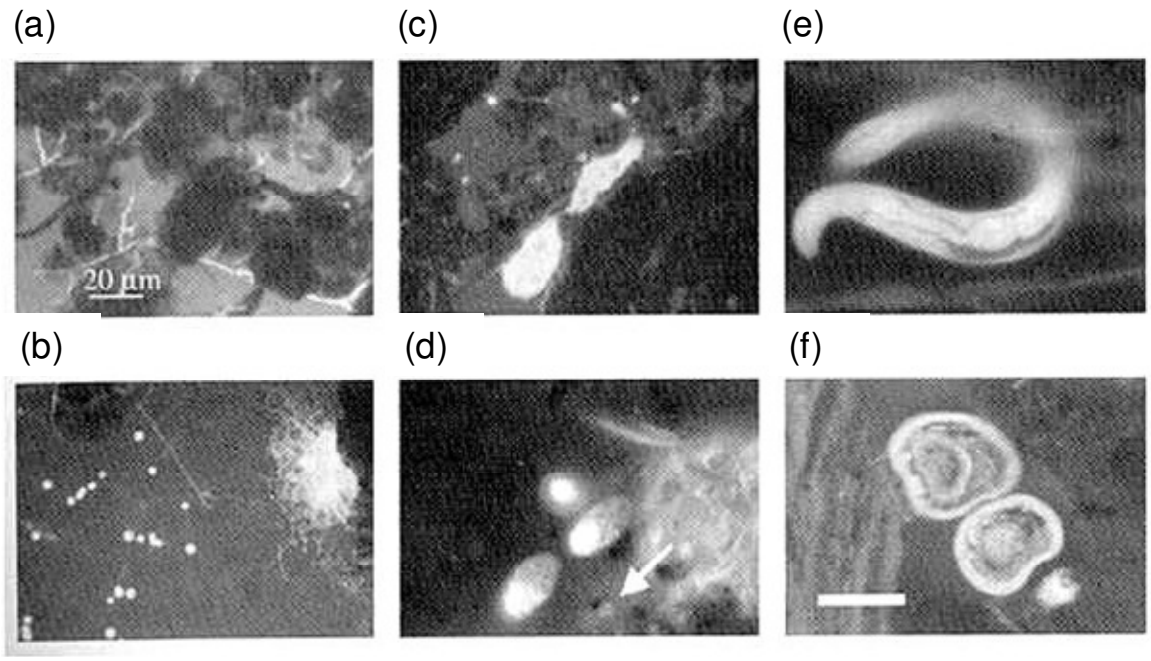


Figure 6 : Les organismes du sol dans leur habitat. Visualisation en coupe mince d'un sol. a/ Mycelium de *Rhizoctonia solani*. b/ Un mycelium non-identifié et des sporanges répartis dans un pore. c/ Une amibe se faufile dans un pore étroit. d/ Amibes à thèque s'accumulant près d'un pore. La flèche désigne une colonie bactérienne. e/ Nématode probablement distordu par la forme du pore qu'il occupe. f/ Coupe tangentielle d'une Enchytraeidae (vers) adjacentes à une racine en décomposition (à gauche). Cette image montre la présence de sol dans le lumen du tube digestif du vers (portion supérieure), et une distorsion du corps de l'animal par les limites du pore. Ces images proviennent d'un même sol. *Source : Young and Ritz (2005).*

Le compartiment sol a longtemps été étudié séparément du compartiment aérien. Cependant, un nombre croissant d'études a permis de mettre en évidence l'étroite relation qu'entretiennent ces deux systèmes (Hooper et al., 2000; Bardgett et al., 2005a; van der Heijden et al., 2008). Comme nous l'avons précédemment évoqué, les microbes du sol influencent le fonctionnement des écosystèmes ainsi que la fitness et la diversité des espèces environnantes (cf §I.3). Mais qu'en est-il des facteurs influençant le compartiment microbien des sols ?

2. Des facteurs abiotiques régulant les communautés microbiennes

L'habitat des micro-organismes du sol est constitué de pores variant dans l'espace (Fig. 6) et le temps, lui conférant une extrême hétérogénéité. La taille et la répartition de ces pores sont essentielles dans la conduction de l'eau et des nutriments, ainsi que dans la communication entre les différentes populations microbiennes (Torsvik and Ovreas, 2002; Young and Ritz, 2005; Standing and Killham, 2007). Ce sont des habitats généralement pauvres et peu favorables au développement de populations microbiennes à forte densité et à activité intense. C'est surtout dans la rhizosphère que les communautés microbiennes se montrent les plus denses et les plus actives (cf §IV.2).

Les mouvements d'eau sont primordiaux pour le maintien de la vie dans le sol. Ils permettent effectivement la diffusion des ions, des nutriments et des gaz dissous, et maintiennent une température favorable aux organismes autochtones. La mobilité de la plupart des micro-organismes étant inféodée à l'eau, ces mouvements d'eau sont également responsables de la dispersion et la connectivité des populations microbiennes. Ces connexions peuvent avoir des effets négatifs, comme une augmentation de la prédation, mais aussi positifs, en augmentant les flux de gènes et les échanges trophiques. Enfin, l'eau influence fortement le pH du sol (Standing and Killham, 2007). Ce pH n'est pas seulement influencé par l'eau, mais aussi par la nature de la roche mère, la qualité de la matière organique, et par l'activité microbienne. Or, le pH a un effet dramatique sur les communautés microbiennes. En effet, basé sur la technique de PLFA (Baath and Anderson, 2003) ont montré une corrélation positive entre le pH du sol et le ratio champignons/bactéries dans des hêtraies chênaies-hêtraies. Basé sur la même technique, l'étude de (Hogberg et al., 2007) le long d'un gradient de pH a mis en évidence une corrélation entre le pH et la biomasse microbienne, positive chez les bactéries, et négative chez les champignons. Les fluctuations de pH induisent également des variations de composition, de structure et de diversité des communautés microbiennes, auxquelles les bactéries apparaissent particulièrement sensibles (Baath and Anderson, 2003; Fierer and Jackson, 2006; Lauber et al., 2008).

La température des sols est également un facteur déterminant dans la distribution et l'activité des micro-organismes, en particulier pour les processus d'ammonification et de nitrification. Cette température affecte non seulement la physiologie des micro-organismes, mais influencent aussi les mouvements d'eau et la diffusion des gaz et des nutriments (Standing and Killham, 2007). Les micro-organismes ne montrent pas tous la même tolérance

face à la température, et on distingue ainsi les psychrophiles supportant des températures allant de -5 à 20 °C ; des mésophiles, qui tolèrent des températures de 15 à 45 °C ; et des thermophiles supportant des températures de 65 à 95°C (Rothschild and Mancinelli, 2001; Standing and Killham, 2007).

D'autres facteurs abiotiques influencent les communautés microbiennes. L'énergie lumineuse a, entre autres, un effet principalement indirect, *via* la stimulation de la germination et de la croissance de la végétation. Elle peut cependant avoir une action directe sur les micro-organismes phototrophes (Standing and Killham, 2007). Enfin, la quantité et la qualité de la matière organique du sol est la source principale d'énergie des communautés microbiennes. La qualité de cette matière organique est très variable, allant de composés facilement dégradables (comme les monomères glucidiques solubles) à d'autres récalcitrants (polymère insolubles comme la lignine). Les caractéristiques de la matière organique peuvent en outre affecter les propriétés physico-chimiques du sol, comme le pH. Cette matière organique est généralement perçue comme une matière morte (*e.g.* litière, fèces, etc…), bien que la biomasse microbienne constitue une part importante de cette matière organique. Provenant d'êtres vivants, ses effets sur les communautés microbiennes seront abordés ci-dessous.

3. Des facteurs biotiques régulant les communautés microbiennes ; le compartiment aérien

Le sol est perpétuellement enrichi par la matière organique provenant des organismes du compartiment aérien, dont les plantes sont les principaux contributeurs (cf §I.3). Ces entrées de ressources sont variables et leur qualité et quantité sont intrinsèquement liées à la nature de la végétation sus-jacente. En effet, les végétaux peuvent être classés selon leurs traits fonctionnels, *i.e.* leurs caractéristiques morphologiques, biochimiques et reproductives (*e.g.* taux de croissance, absorption des nutriments, dégradabilité...). Ces traits co-varient avec les conditions environnementales par effet de rétroaction (*i.e.* réponse et effet, revue dans [Eviner and Chapin, 2003]). A l'échelle de la communauté, le type de réponses/effets face aux contraintes environnementales est principalement porté par les espèces dominantes qui constituent une part importante de la biomasse (Grime, 1998). Ainsi, les contraintes environnementales et les groupes fonctionnels dominants déterminent le fonctionnement des écosystèmes, notamment à travers la production primaire et la décomposition (Grime, 1998; Lavorel and Garnier, 2002).

Ces traits fonctionnels influencent donc la qualité et la quantité de litière (Fig. 7a). Or, la qualité des ressources du sol influence de manière significative les communautés microbiennes (Zhou et al., 2002; Wardle et al., 2004; Chapman et al., 2006; Lauber et al., 2008). Ainsi, plusieurs études ont mis en évidence l'effet de l'utilisation des terres ou du couvert végétal sur les communautés microbiennes (Fierer and Jackson, 2006; Yao et al., 2006; Zak and Kling, 2006; Kasel et al., 2008; Lauber et al., 2008). De plus, l'augmentation de la récalcitrance de la matière organique provoque une diminution de la biomasse microbienne, et pourrait parallèlement résulter en une augmentation de la diversité fonctionnelle de ces communautés (Hopkins and Gregorich, 2005). Cependant, l'impact de la qualité de la litière, et indirectement celui de la diversité fonctionnelle des communautés végétales, sur la diversité et le recrutement des espèces microbiennes restent encore mal caractérisés.

La productivité du milieu, ou l'apport des ressources, régulent la diversité des macro-organismes. Cette relation est unimodale, et le maximum de diversité est atteint à des niveaux intermédiaires de productivité. Il semble que cette relation productivité-diversité soit similaire chez les micro-organismes tant bien en milieu aquatique (Horner-Devine et al., 2003), qu'en milieu terrestre (Bardgett et al., 2005a; Waldrop et al., 2006, Fig. 7b).

La composition de la végétation peut aussi influencer les communautés microbiennes, particulièrement celles associées aux racines soit par leur capacité à sécréter des métabolites secondaires antimicrobiens, soit par leur vulnérabilité face aux pathogènes, soit enfin par les associations qu'elles établissent avec les micro-organismes, comme les mycorhizes. Cependant, l'association entre une espèce hôte et une espèce microbienne est rarement obligatoire (Allen et al., 1995; Gardes and Dahlberg, 1996) et il est difficile de statuer sur l'existence d'une relation entre la diversité en espèce des communautés végétales, et celle des micro-organismes du sol. En effet, bien qu'il semble exister une relation entre la diversité des communautés végétales et celle des communautés microbiennes (Wardle et al., 2004), cette relation dépend du contexte de l'étude. Par exemple, Waldrop et al. (2006) (Fig. 7b), n'ont pas observé d'effet direct de la diversité des communautés végétales sur la diversité des champignons du sol. De la même manière, une seule espèce d'arbre peut favoriser la diversité microbienne en rendant le milieu beaucoup plus hétérogène en termes de structure et de ressources qu'une pelouse diversifiée (revue dans Hooper et al., 2000).

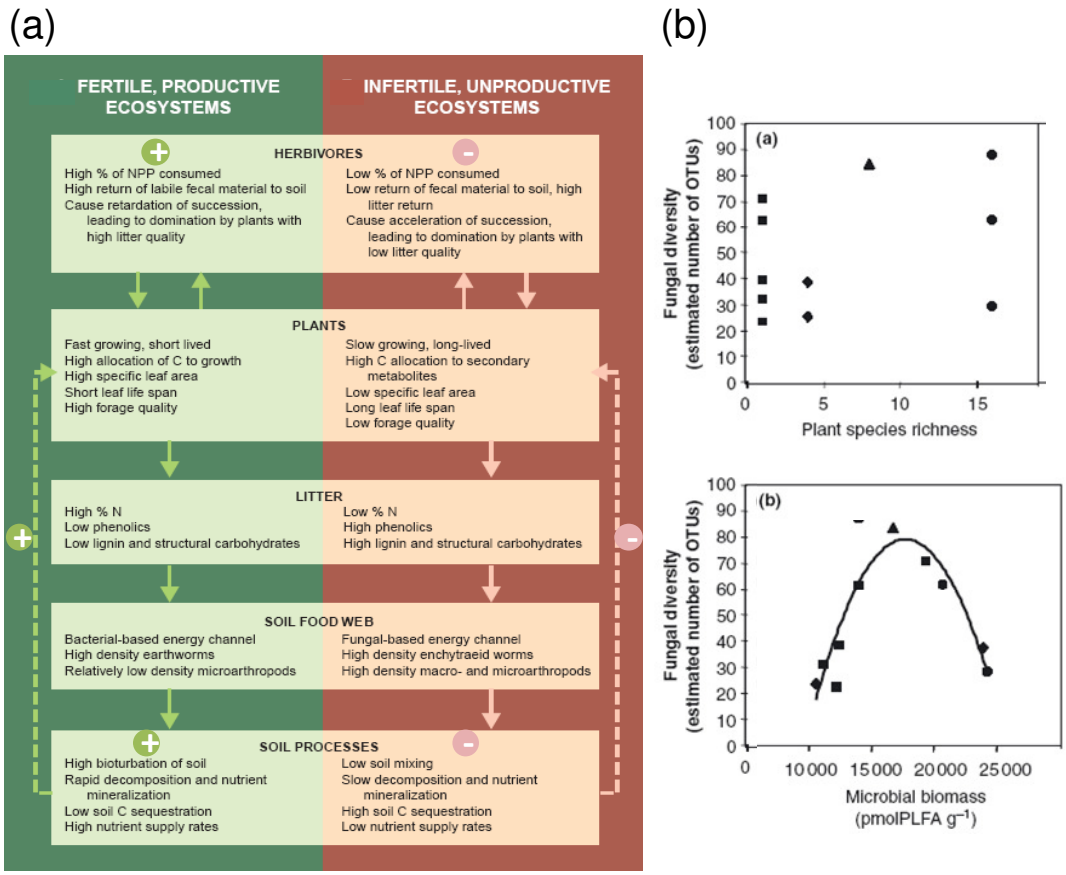


Figure 7 : La nature des communautés végétales influence la disponibilité des ressources et le fonctionnement des sols. **(a)** les espèces à croissance rapide ont une litière plus facilement dégradable, favorisant la biomasse bactérienne. Ces espèces sont également soumises à d'avantage d'herbivorie. Ces systèmes sont donc fertiles et les processus de recyclage de la matière organique sont rapides. *A contrario*, les espèces à croissances lentes ont une litière récalcitrante, moins accessible aux bactéries. La biomasse fongique est donc plus elevée mais les processus de recyclage de la matière organique restent faibles. *D'après Wardle et al. (2004).* **(b)** la diversité des communautés végétales ne semble pas affecter la diversité fongique, mais celle-ci apparaît clairement dépendante de la productivité du milieu (ici assimilée comme étant la biomasse microbienne). *D'après Waldrop et al. (2006).*

La végétation influence aussi ces communautés à une échelle locale, dans la rhizosphère. En effet, les racines des plantes contribuent à la structuration du sol, se renouvèlent en permanence, et produisent des exsudats (*e.g.* carbohydrates, acides organiques, acides aminés) dont la qualité et la production sont variables dans le temps et selon la plante, (Bardgett et al., 2005b; Bais et al., 2006; Standing and Killham, 2007). En effet, le stade de développement de la plante et la rhizodéposition ont un impact significatif sur la structure des communautés microbiennes de la rhizosphère de *Medicago truncatula*, *Chrysanthemum* et *Lolium multiflorum* (Duineveld et al., 2001; Butler et al., 2003; Mougel et al., 2006).

Enfin, les ressources du sol sont utilisées par des acteurs divers. Parmi ces ressources, l'azote est le plus abondamment documenté en raison de son importance dans la croissance des végétaux. Or, il existe une compétition entre les plantes et le compartiment sous-terrain pour cette ressource, comme l'ont montré entre autres les études de (Lipson et al., 1999) et (Jonasson et al., 1999) qui peuvent réduire la productivité des plantes (van der Heijden et al., 2008). Les relations entre les communautés végétales et les micro-organismes du sol sont abordées dans les Chapitres 2 et 3 du présent manuscrit.

4. Des facteurs biotiques régulant les communautés microbiennes ; le compartiment sous-terrain

Les communautés microbiennes du sol sont également fortement influencées par les interactions qu'entretiennent les espèces du compartiment souterrain. Ces interactions ont lieu à une échelle bien inférieure à celle des animaux ou des végétaux. En effet, la structure du sol partitionne les communautés microbiennes dans des micro-niches. Ces « îlots » de populations microbiennes peuvent être connectés par des mouvements d'eau, par la croissance de racines des plantes ou celle du mycélium fongique, et par la mobilité de la faune du sol. **La fragmentation des communautés microbiennes du sol et leur degré de connectivité sont déterminantes dans leurs interactions** physiques, biochimiques, et trophiques (Encadré 4, Van Elsas et al., 2007).

Les interactions biotiques sont habituellement classées le long d'un continuum entre les bénéfices et les pertes résultant de l'interaction (Fig. 8). Précisons ici que la nature de ces interactions n'est pas toujours fixe, et qu'elle peut se déplacer le long du continuum parasitisme/mutualisme au cours de l'existence des protagonistes de l'interaction (Johnson et al., 1997). Dans la catégorie des interactions antagonistes se classent la prédation et le parasitisme, dans lesquels l'un des protagonistes gagne un avantage aux dépens de l'autre. Citons par exemple les champignons trappeurs de nématodes (Fig. 8 ; Van Elsas et al., 2007), la mycophagie bactérienne (revue dans de Boer et al., 2005), ou l'infection d'une population bactérienne par des bacteriophages. Ces interactions peuvent être cycliques puisqu'elles peuvent entraîner une diminution de la densité d'hôtes/proies et ainsi réduire la densité de parasite/prédateurs (Van Elsas et al., 2007).

Encadré 4 : Quorum sensing

Les ilots de populations microbiennes, constitués d'une ou plusieurs espèces, forment une matrice liée par une quantité plus ou moins importante d'exopolymères extracellulaires d'origine bactérienne ou fongique, appelée biofilm. A l'intérieur de ces matrices, chaque cellule est susceptible de percevoir et d'interagir avec les individus voisins (Van Elsas et al., 2007).

Le quorum sensing correspond aux mécanismes qui coordonnent le comportement d'une population en fonction de sa densité. Chez les micro-organismes, cette coordination se fait *via* la production/perception de molécules, appelées auto-inducteurs, dans le biofilm. Ces auto-inducteurs, comme les lactones homoserine acétylées (bactéries) et le farnésol (champignons) agissent en régulant l'expression de certains gènes (Hogan, 2006; Van Elsas et al., 2007).

La densité de la population détermine la concentration des auto-inducteurs dans le milieu. Au sein d'une population spécifiquement homogène, le quorum sensing permet par exemple l'activation de gènes de virulence lorsque la population est suffisamment dense pour infecter son hôte, comme chez *Pseudomonas aeruginosa* ou *Candida albicans*. Ces molécules peuvent également avoir un rôle dans la compétition en agissant de la même manière que les antibiotiques (Hogan, 2006; Van Elsas et al., 2007).

Enfin, une multitude d'interactions chimiques ont lieu dans le sol et sont impliquées dans de nombreux processus écologiques. Citons par exemple l'implication des phéromones dans la reproduction sexuée chez les champignons (Hogan, 2006; Van Elsas et al., 2007).

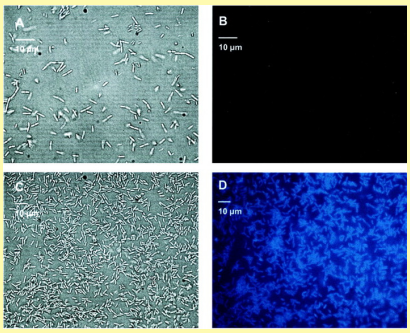


Figure E4 : Initiation de la détéction du quorum pendant le développement d'un biofilm de la souche *Pseudomonas aeruginosa* PAO230. Visualisation par microscopie de : A, la phase réversible d'attachement du développement du biofilm ; B, la même phase sous lumière UV indique un niveau de détection du quorum nul; C, la phase irréversible d'attachement du développement du biofilm, D, la même phase sous lumière UV indique un niveau de détection du quorum élevé. *D'après Sauer et al. (2002).*

Ces interactions antagonistes comprennent aussi la compétition pour l'occupation de l'espace et l'exploitation des ressources. Par exemple, les bactéries et les champignons sont les acteurs principaux de la dégradation de la matière organique du sol et constituent la base vivante des chaînes trophiques du sol (Bardgett, 2005). Il existe donc un recouvrement entre les niches bactériennes et fongiques. En conséquence, ces deux taxons se mènent une « guerre froide », où la diminution de la croissance de l'un entraîne une augmentation de la croissance de l'autre (de Boer et al., 2005; Rousk et al., 2008). Cette compétition s'effectue principalement par la production respective d'antibiotiques ou d'inhibiteurs de croissance (phénomènes appelés Fongistase et Bactériostase ; Fig. 8), qui peuvent interférer avec la synthèse de la paroi cellulaire ou de protéines ou encore affecter l'intégrité de la membrane cellulaire ou le métabolisme de l'acide nucléique (Van Elsas et al., 2007). Notons que ces phénomènes de compétition sont impliqués dans la spécialisation aux niches écologiques chez

des populations de *Pseudomonas fluorescens,* et ont donc un effet potentiellement positif sur la diversité microbienne des sols (Rainey et al., 2005).

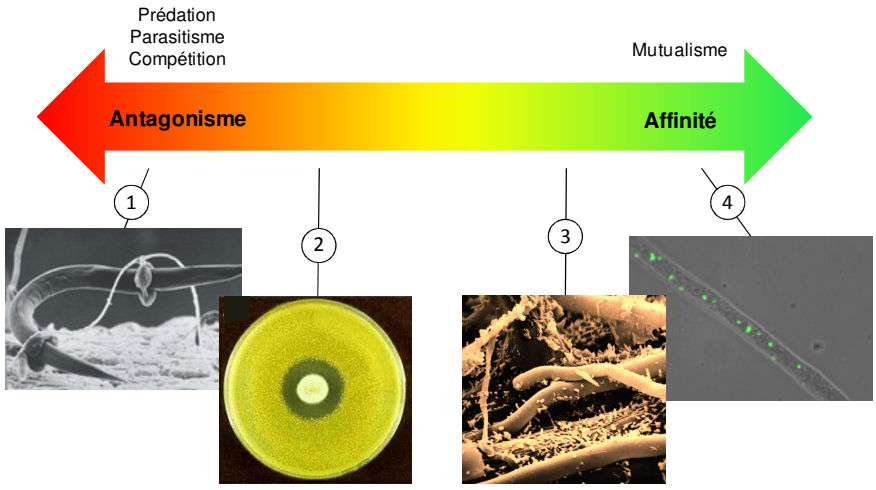


Figure 8 : Les interactions microbiennes dans le sol. 1/ Visualisation par microscopie électronique à balayage d'un champignon se nourrissant d'un nématode. *Photographie : G.L. Barron.* **2/** Photographie de l'inhibition de la croissance de *Micrococcus luteus* (en jaune) par la production de pénicilline par *Penicillium notatum* (au centre). *Source : http://www.biology.ed.ac.uk/research/groups/jdeacon /microbes/penicill.htm.* **3/** Visualisation par microscopie électronique à balayage de bactéries réparties le long d'hyphes fongiques. *Source : http://soils.usda.gov/sqi/concepts/soil_biology/bacteria.html.* **4/** Visualisation par microscopie confocale à balayage laser de l'endosymbionte bactérien marqué à la GFP (*Burkholderia* sp.) dans un hyphe de *Rhizopus microsporus*. *Source : Partida-Martinez et al. (2007).*

Parallèlement, les micro-organismes du sol peuvent entretenir des relations positives et plus ou moins obligatoires. Par exemple, les phénomènes de facilitation sont courants et se retrouvent typiquement dans les relations trophiques. Cette facilitation s'opère lorsqu'un taxon modifie le milieu de façon avantageuse pour d'autres sans pour autant affecter sa propre fitness. Dans ce contexte, la dégradation de la matière organique résulte d'une coopération métabolique entre les populations microbiennes, où certaines d'entre elles sont capables de métaboliser un composé dont les métabolites peuvent être utilisés par cette même population et/ou par d'autres. Ce type d'interaction est courant entre bactéries et champignons (Fig. 8) puisque ce sont ces derniers qui sont principalement impliqués dans la dégradation de substrats complexes (*e.g.* lignocellulosiques, de Boer et al., 2005). En outre, des études ont montré des cas de stimulation de la germination de spores fongiques par certaines bactéries. Parmi les différents mécanismes impliqués, citons l'activité chytinolytique de bactéries dégradant la paroi de la spore fongique (revue dans de Boer et al., 2005).

Enfin, certaines populations microbiennes entretiennent des relations obligatoires dont les deux protagonistes tirent bénéfice. Par exemple, l'un des facteurs de virulence du champignon pathogène du riz, *Rhizopus microsporus*, provient des bactéries qu'il abrite (Fig. 8, Partida-Martinez et al., 2007). De même, de nombreux champignons mycorhiziens arbusculaires abritent des endosymbiontes bactériens du genre *Burkholderia* possédant des gènes codant pour la nitrogénase, enzyme responsable de la fixation de l'azote atmosphérique. Cette symbiose consisterait en un échange d'éléments azotés fournis par la bactérie contre un apport de phosphore provenant du champignon (Minerdi et al., 2002).

La composition et la diversité des communautés microbiennes des sols résultent de facteurs et d'interactions multiples mais dont les mécanismes restent encore très mal caractérisés. Une meilleure caractérisation de la relation diversité/fonction entre les compartiments aérien/souterrain, tout comme entre les populations microbiennes est donc nécessaire pour comprendre les mécanismes impliqués dans l'assemblage et la succession des communautés microbiennes.

V. Le cas particulier des écosystèmes alpins

1. *Présentation du système*

En biogéographie, l'étage alpin se définit comme étant un milieu d'altitude se situant entre l'étage subalpin et l'étage nival (Fig. 9). Ces écosystèmes se caractérisent par des moyennes de températures annuelles faibles et une végétation du type « toundra » qui se distingue par l'absence d'arbres et par des espèces de basse stature. Ces écosystèmes sont généralement associés aux toundras arctiques, réparties dans les hautes latitudes, qui présentent des caractéristiques similaires (Körner, 1995). Les écosystèmes alpins recouvrent près de 3% de la surface terrestre du globe, et se retrouvent principalement dans l'hémisphère Nord, mais aussi en Afrique centrale, en Nouvelle-Zélande, et dans les Andes. Les toundras alpines sont en outre soumises à des froids intenses, de fortes radiations solaires, sont particulièrement exposées aux vents, et sont des milieux relativement pauvres en nutriments (Körner, 1995). Ces conditions drastiques recrutent des espèces résistantes au froid, dont les arbres sont exclus.

Figure 9 : Les écosystèmes alpins. Etagement de la végétation dans le Dauphiné et fragmentation du paysage alpin. *Source : Station Alpine Joseph Fourier*

L'étage alpin est un étage de **contrastes**. A large échelle, ces contrastes sont façonnés par l'histoire des sites, soumis aux glaciations et aux tectoniques des plaques. Ces phénomènes résultent en une **fragmentation du paysage**, dont la plus notable est celle de l'exposition à l'ensoleillement (Fig. 9). Ces écosystèmes connaissent également de grands écarts thermiques et sont soumis à un rythme saisonnier contrasté (Körner, 1995).

La fragmentation du paysage alpin résulte d'une forte hétérogénéité méso-topographique, entraînant une variabilité de la distribution des radiations, de l'eau et du manteau neigeux (Fig. 9) sur de petites distances. Or, la durée du manteau neigeux est déterminante dans la longueur de la saison de végétation, comme l'intensité des radiations et la disponibilité en eau le sont pour la croissance des végétaux (Walker, 1995). La présence ou l'absence de ce manteau neigeux a également des conséquences dramatiques sur la température des sols durant la saison hivernale. Ainsi, ces variations topographiques, souvent assimilées au gradient d'enneigement, ont un impact considérable sur les conditions édapho-climatiques (Litaor et al., 2001), sur la composition des communautés végétales (Choler, 2005) et sur les processus ecosystémiques (Olear and Seastedt, 1994; Fisk et al., 1998; Hobbie et al., 2000; Edwards et al., 2007). En conséquence, les écosystèmes alpins constituent une **mosaïque d'habitats** abritant une végétation diverse en terme d'espèces et de fonctions (Hobbie, 1995; Körner, 1995).

Les sols de ces écosystèmes arctico-alpins séquestrent de grandes quantités de carbone sous forme organique. Cette accumulation de matière organique résulte d'un déséquilibre entre les flux de carbone entrant dans le sol, via la photosynthèse, et les flux sortant, via la respiration des racines et des micro-organismes. Ce déséquilibre provient des températures faibles qui réduisent de manière significative l'activité microbienne, et par conséquent les processus de dégradation de la matière organique (Davidson and Janssens, 2006). Ces réservoirs de carbone sont donc potentiellement vulnérables dans un contexte de réchauffement climatique, mais leur devenir reste indéfini en raison de la complexité des processus de dégradation de la matière organique (Hobbie et al., 2000).

Cependant, le statut « stock de carbone » des sols alpins doit être nuancé. En effet, Schinner (1983) a mis en évidence un effet de la topographie et des saisons sur les flux de carbone et les activités microbiennes. De plus, (Brooks et al., 1997) ont rapporté que les systèmes présentant un enneigement en hiver montrent des pertes de CO_2 plus importante que dans les systèmes présentant un enneigement faible, d'autant plus si cet enneigement est tardif. De la même manière, (Monson et al., 2006) ont mis en évidence un effet significatif de la quantité d'enneigement sur les pertes de CO_2 des sols de forêts sub-alpines (Fig. 10). Or, l'enneigement des écosystèmes alpins est fortement hétérogène. Associé à cela, les communautés végétales alpines ne montrent pas les mêmes traits de réponses aux régimes d'enneigement, notamment en terme de qualité de litière (Choler, 2005; Baptist, 2008, Fig. 10). Les micro-organismes étant des acteurs principaux dans le recyclage du carbone du sol et

dépendant des ressources et de la température, leur activité est donc susceptible d'être influencée par le couplage qualité de litière/enneigement. Il est donc probable que les écosystèmes alpins présentent à la fois des zones « sources » et des zones « puits » de carbone le long du gradient d'enneigement (Fig. 10). Or le comportement de la composante microbienne de ces systèmes, jusqu'ici principalement appréhendé *via* des mesures de flux de carbone, reste mal caractérisé.

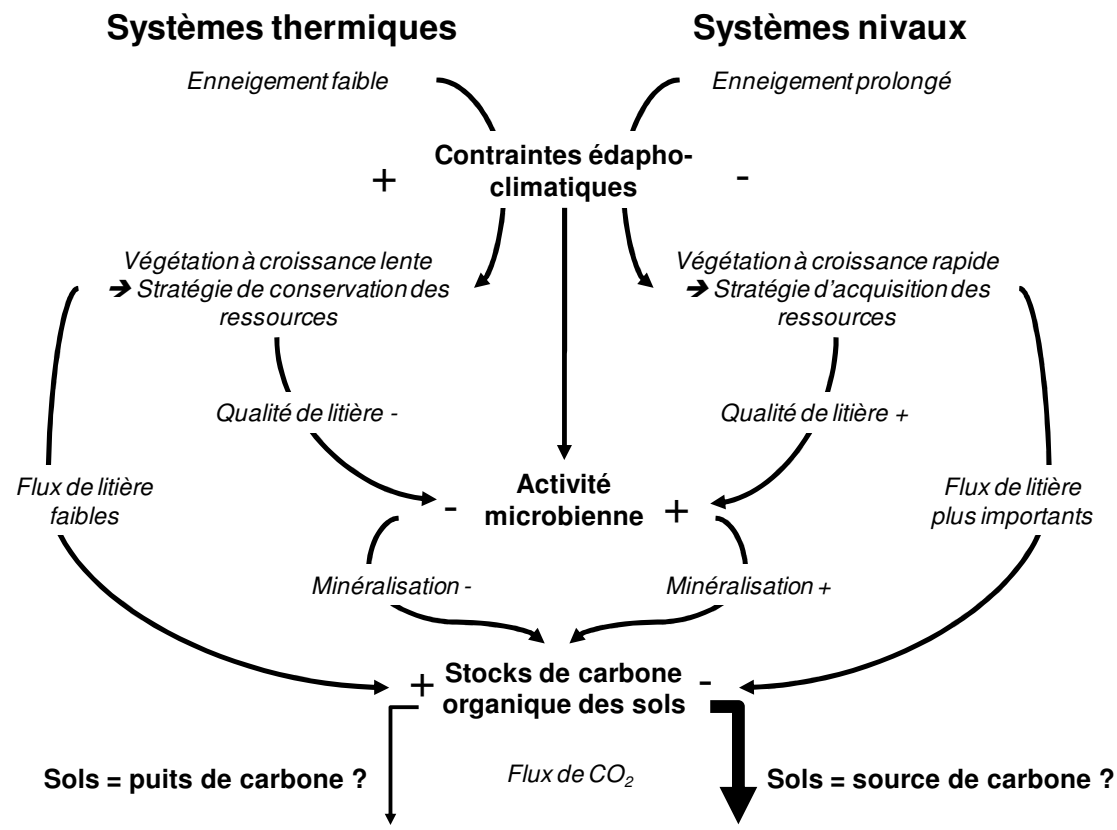


Figure 10 : Hypothèse du comportement source *vs.* puits de carbone des sols alpins en relation avec les régimes d'enneigement. Les conditions édapho-climatiques locales, déterminées par le régime d'enneigement réguleraient les flux de carbones locaux en affectant de façon directe ou indirecte les communautés végétales et les communautés microbiennes. *D'après Baptist (2008).*

2. Les micro-organismes de l'étage alpin

La recherche de souches psychrophiles, dont les applications industrielles sont diverses (Rothschild and Mancinelli, 2001), couplée aux questionnements du comportement des micro-organismes face au réchauffement global, ont motivé l'étude des communautés

microbiennes arctico-alpines. Dans le contexte du réchauffement climatique, ces communautés ont surtout été abordées par mesure de flux de CO_2 et de nutriments. Ainsi, la biomasse microbienne et l'utilisation des nutriments par les micro-organismes sont positivement corrélées au gradient d'enneigement (Fisk et al., 1998). Cependant, l'exposition des sols alpins à des températures plus élevées n'a pas montré d'effets particuliers sur cette biomasse (Jonasson et al., 1999). Une dynamique saisonnière a également été mis en évidence en termes de flux de carbone et d'activités enzymatiques, particulièrement due aux conditions hivernales, sous manteau neigeux (Lipson et al., 1999; Hobbie et al., 2000; Monson et al., 2006).

La composition des communautés microbiennes alpines ont surtout été abordées par des techniques classiques, et particulièrement portées sur les champignons mycorhiziens de par leur implication dans la fitness de la végétation et dans le cycle de l'azote (Read and Haselwandter, 1981; Gardes and Dahlberg, 1996; Cripps and Eddington, 2005). Ces études ont apporté des informations significatives sur la répartition et la dominance de certains types d'associations mycorhiziennes (*e.g.* arbusculaire, ectomycorhizienne) selon la plante ou l'altitude. D'autres études ont mis en évidence l'effet du gradient d'enneigement et/ou de la saison sur la structure des communautés microbiennes (Zak and Kling, 2006; Bjork et al., 2008), mais demeurent peu informatives car reposent principalement sur des empreintes PLFA (cf. §II.2).

L'appréhension moléculaire des communautés microbiennes alpines a principalement été initiée par l'équipe de Steven K. Schmidt, de l'université du Colorado. Leurs précédentes études menées sur le cycle de l'azote couplé à la biomasse et à l'activité microbienne ont permis d'établir un modèle du fonctionnement annuel de ces écosystèmes (revue dans (Bardgett et al., 2005b; Schmidt et al., 2007) ; Fig. 11). L'approche moléculaire appliquée à ces systèmes a mis en évidence une **variation saisonnière** significative de la composition en espèces des communautés bactériennes (Lipson and Schmidt, 2004) et fongiques (Schadt, 2002; Schadt et al., 2003) dans des sols de pelouses dominées par *Kobresia myosuroides* (revue dans Nemergut et al., 2005). Plus particulièrement, (Schadt et al., 2003) ont mis en évidence un nouveau lignage de champignons principalement présent sous manteau neigeux. De même, le développement de certains champignons appartenant au phylum des Zygomycetes a été observé à la fonte des neiges (Schmidt et al., 2008). Cependant, l'ensemble de ces études a rarement pris en compte la fragmentation du paysage alpin. Cette thèse présente dans le Chapitre II un suivi temporel des communautés microbiennes de deux

écosystèmes se situant aux deux extrêmes du gradient d'enneigement, et une étude à l'échelle du bassin versant dans le Chapitre III.

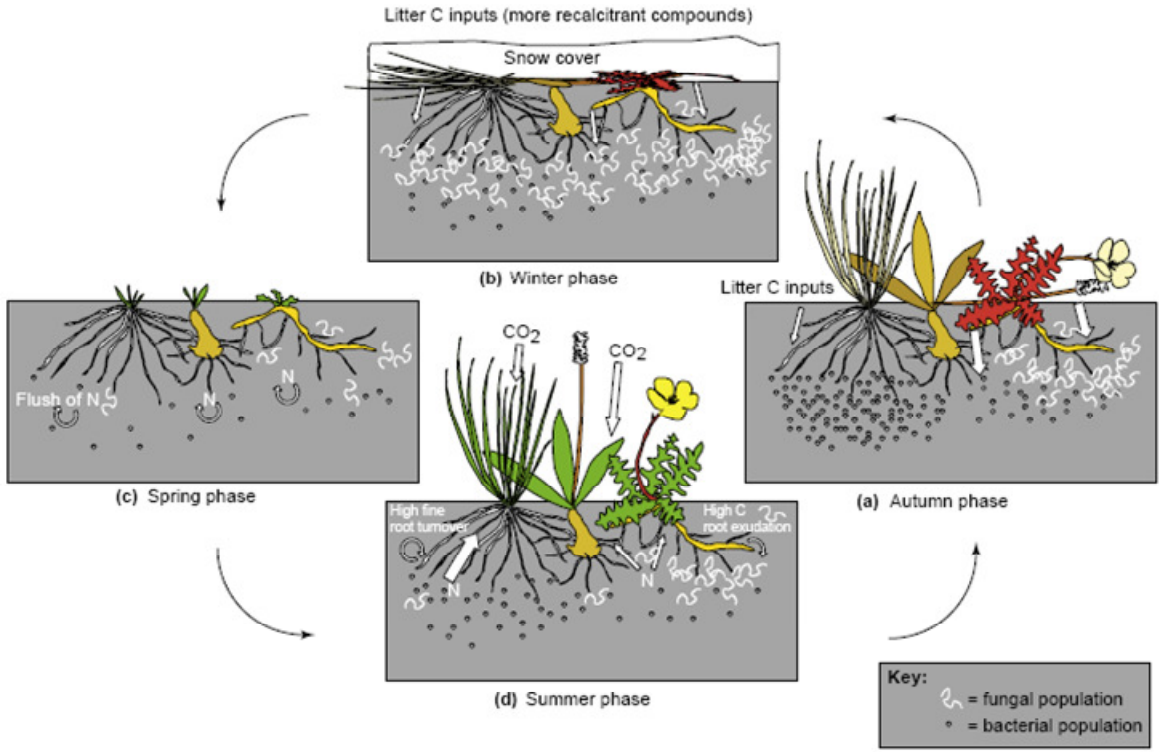


Figure 11 : Dynamique saisonnière des échanges de ressources entre plantes et micro-organismes du sol. **(a)** Pendant l'automne, la sénescence de la végétation génère des entrées de carbone labile dans le sol. Ces entrées de carbone favorisent plus ou moins la croissance et la diversité des populations microbiennes selon la qualité et la quantité de la litière. **(b)** En hiver, la biomasse microbienne poursuit sont travail de décomposition sous le manteau neigeux. Au fur et à mesure de l'utilisation des ressources, les molécules les plus récalcitrantes promeuvent la dominance des champignons. **(c)** Le réchauffement du climat au printemps provoque un renouvellement rapide des populations microbienne, libérant une quantité importante d'azote labile disponible pour les plantes. **(d)** L'azote est prélevé par les plantes pour leur croissance au début de l'été. Le carbone atmosphérique piégé par les plantes est quant à lui partiellement réalloué vers le sol par renouvellement ou rhizodéposition des racines. *Source : Bardgett et al. (2005b)*

Les écosystèmes alpins sont donc des paysages fragmentés qui montrent une diversité large d'habitats et contrastés par l'enneigement et les dynamiques saisonnières. Ce type de milieu permet donc de tester sur des pas de temps et de distances relativement courts l'effet de l'environnement et de sa dynamique sur les communautés microbiennes. Leur étude est d'autant plus nécessaire dans un contexte de perte de la biodiversité et de réchauffement climatique.

Chapitre I - Optimisation de la CE-SSCP pour l'analyse des communautés microbiennes

I. Problématique et démarche scientifique

1. Contexte général

Etudier l'impact des changements environnementaux sur les communautés microbiennes nécessite l'analyse d'un grand nombre d'échantillon. Comme nous l'avons précédemment évoqué, la structure génétique de ces communautés est maintenant classiquement appréhendée (i) en extrayant l'ADN total d'un échantillon environnemental, (ii) en amplifiant par PCR un marqueur moléculaire, et (iii) en déterminant la variabilité de ce marqueur à l'aide de techniques d'empreintes moléculaires (cf. Introduction §II.4 ;Fig. 12a). Brièvement, ces techniques consistent à séparer les fragments d'ADN d'un mélange complexe à l'aide d'un gel polyacrylamide soumis à un champ électrique et dont les conditions sont dénaturantes ou natives. L'ADN étant chargé négativement, la vitesse de migration des fragments dans le gel dépend de leur taille et/ou de leur séquence nucléotidique (Fig. 12b).

Il est maintenant admis que chacune des étapes expérimentales conduisant à la caractérisation de la structure de ces communautés est soumise à plusieurs types de biais (cf. Introduction §II.4). Par exemple, l'extraction d'ADN peut être incomplète et l'amplification par PCR peut générer des erreurs. Ces biais peuvent avoir des répercussions significatives sur la qualité des profils moléculaires et sur leur interprétation puisqu'ils peuvent soit exclure certains groupes microbiens (extraction et PCR), soit générer des fragments d'ADN dépourvus de sens biologique (PCR). Ainsi, afin de **refléter au mieux la structure des communautés microbienne** *via* ces techniques, il est nécessaire d'établir un **compromis** entre les biais des différentes étapes précédant l'électrophorèse, les conditions électrophorétiques (cf Introduction §II.4, Fig. 12), et la nécessité de disposer d'une technique à haut-débit.

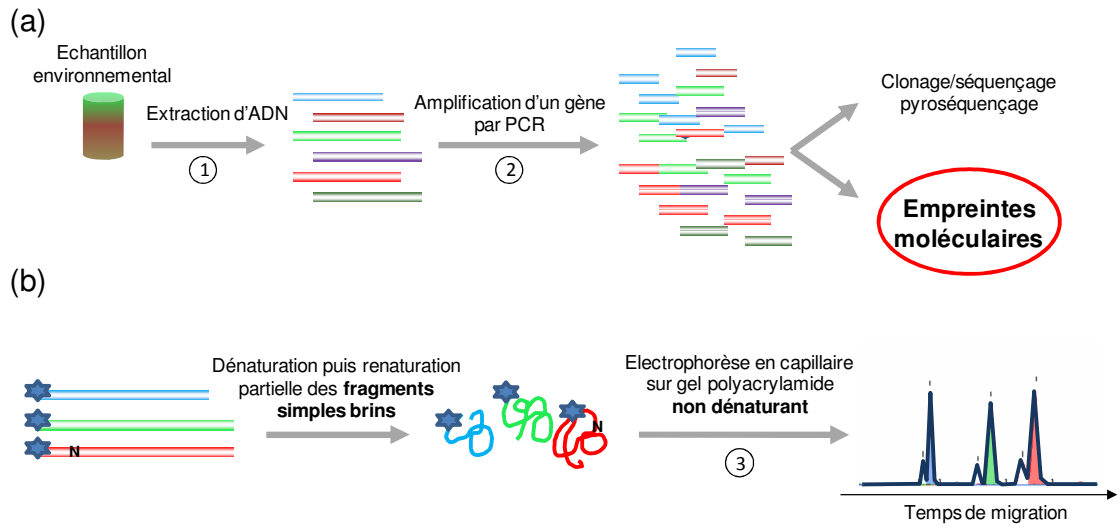


Figure 12 : (a) Démarche expérimentale classique pour l'étude des communautés microbiennes. (b) Principe de la CE-SSCP. Les fragments d'ADN simples brins marqués par un fluorophore sont séparés par électrophorèse selon leur taille et leur composition (N : mutation ponctuelle). Les fragments en fin de migration sont détectés par fluorescence. Les numéros correspondent aux étapes expérimentales optimisées dans le présent chapitre.

Comme toutes techniques d'empreintes moléculaires, la SSCP (Single Strand Conformation Polymorphism) repose sur la séparation de brin d'ADN selon leur taille, mais permet aussi de mettre en évidence des variations de séquences. En effet, sous conditions non dénaturantes, l'ADN simple brin adopte une conformation tridimensionnelle dépendant de sa composition nucléotidique et de son environnement physico-chimique (Schwieger and Tebbe, 1998, Fig. 12b). Initialement développée dans le but de détecter des mutations ponctuelles (SNP, Single Nucleotide Polymorphism) responsables de maladies génétiques humaines (Orita et al., 1989), la SSCP a ensuite été appliquée au typage de souches bactériennes dans un cadre clinique, puis à l'analyse de communautés bactériennes complexes en raison de sa simplicité d'application par rapport à d'autres méthodes, tel que la DGGE (Denaturing Gradient Gel Electrophoresis, Schwieger and Tebbe, 1998).

2. Objectifs de l'étude

Disposant d'un séquenceur capillaire au laboratoire, nous avons choisi d'y appliquer la méthode SSCP. En effet, la longueur des capillaires dont nous disposions permettaient difficilement d'utiliser des techniques d'empreintes moléculaires générant des fragments d'ADN de plus de 700 paires de bases (bp), comme l'ARDRA (Amplified Ribosomal DNA Restriction Analysis) ou l'ARISA (Automated rRNA Intergenic Spacer Analysis). Il nous

était également impossible d'appliquer un gradient de température ou de dénaturation, bannissant l'utilisation de la DGGE ou la TGGE. Enfin, bien que la T-RFLP soit décrite comme plus performante pour l'analyse des communautés microbiennes (Tiedje et al., 1999), notre choix s'est porté sur la SSCP, pour favoriser le haut débit. En effet, contrairement à la T-RFLP, la SSCP ne nécessite pas d'étape de digestion, limitant les expérimentations en termes de coûts et de temps. Dans ce sens, l'électrophorèse en capillaire permet une augmentation du nombre d'échantillons analysés, mais aussi de la ségrégation et la détection des fragments. En effet, les fragments d'ADN peuvent être discriminés à la paire de base près contrairement aux gels classiques dont la résolution est moindre.

Dans un souci d'optimisation de la SSCP pour un criblage à haut débit des communautés microbiennes, nous avons testé les différents facteurs ayant un impact potentiel sur la qualité des profils obtenus. Ce chapitre s'articule donc autour de :

1. L'optimisation de la CE-SSCP pour l'étude des communautés bactériennes. En utilisant la région V3 du gène de l'ARNr 16S, nous avons testé la reproductibilité de la CE-SSCP et l'impact de l'extraction d'ADN et de la PCR (*e.g.* conditions PCR, quantité d'ADN ou type d'ADN polymérase) sur la qualité des résultats obtenus (Article A, Annexe A).

2. L'application de cette méthode à l'étude des communautés fongiques en utilisant la région ITS1. Cette étude a également consisté à appréhender (i) l'impact de la température de migration sur la discrimination des fragments et (ii) l'efficacité de la CE-SSCP par rapport à la CE-FLA, une techniques discriminant les fragments uniquement selon leur taille (Article B).

II. Contribution scientifique

Article A: Zinger L., Gury J., Giraud F., Krivobok S., Gielly L., Taberlet P., and Geremia R.A. (2007) Improvements of polymerase chain reaction and capillary electrophoresis single-strand conformation polymorphism methods in microbial ecology: Toward a high-throughput method for microbial diversity studies in soil. *Microb. Ecol.* **54**: 203-216.

Annexe A: Gury J., Zinger L., Gielly L., Taberlet P., and Geremia R.A. (2008) Exonuclease activity of proofreading DNA polymerases is at the origin of artifacts in molecular profiling studies. *Electrophoresis* **29**: 2437-2444.

Article B: Zinger L., Gury J., Alibeu O., Rioux D., Gielly L., Sage L. et al. (2008) CE-SSCP and CE-FLA, simple and high-throughput alternatives for fungal diversity studies. *J. Microbiol. Meth.* **72**: 42.

Chapitre I – Article A:

Improvements of PCR and CE-SSCP methods in microbial ecology: towards a high-throughput method for microbial diversity studies in soil.

Lucie Zinger, Jérôme Gury, Frédéric Giraud, Serge Krivobok, Ludovic Gielly, Pierre Taberlet & Roberto A. Geremia.

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Microbial Ecology

Improvements of Polymerase Chain Reaction and Capillary Electrophoresis Single-Strand Conformation Polymorphism Methods in Microbial Ecology: Toward a High-throughput Method for Microbial Diversity Studies in Soil

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Abstract

The molecular signature of bacteria from soil ecosystems is an important tool for studying microbial ecology and biogeography. However, a high-throughput technology is needed for such studies. In this article, we tested the suitability of available methods ranging from soil DNA extraction to capillary electrophoresis single-strand conformation polymorphism (CE–SSCP) for high-throughput studies. Our results showed that the extraction method does not dramatically influence CE–SSCP profiles, and that DNA extraction of a 0.25 g soil sample is sufficient to observe overall bacterial diversity in soil matrices. The V3 region of the 16S rRNA gene was amplified by PCR, and the extension time was found to be critical. We have also found that proofreading DNA polymerases generate a better signal in CE–SSCP profiles. Experiments performed with different soil matrices revealed the repeatability, efficiency, and consistency of CE–SSCP. Studies on PCR and CE–SSCP using single-species genomic DNA as a matrix showed that several ribotypes may migrate at the same position, and also that single species can produce double peaks. Thus, the extrapolation between number of peaks and number of species remains difficult. Additionally, peak detection is limited by the analysis software. We conclude that the presented method, including CE–SSCP and the analyzing step, is a simple and effective technique to obtain the molecular signature of a given soil sample.

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Introduction

Soil microorganisms play a significant role in above-ground ecosystems, by influencing plants in either a positive (nutritional) or negative (pathogenic) way [13, 34, 39], and are also involved in central biogeochemical cycles (nitrogen, carbon, and phosphorus). The precise description of the bacterial diversity of a given sample is not necessary to understand microbial function in the ecosystem, but there is a capital interest to develop high-throughput methods allowing the simultaneous study of many samples. Indeed, spatiotemporal dynamics of bacterial communities, evaluation of the impact of pollutants, or study of biogeography of plant/microbial associations require frequent processing of multiple samples. Methods that gather molecular signatures are an important tool for the study of soil microbial dynamics, both as a method of describing bacterial diversity and for biogeography studies.

In bacterial diversity studies, molecular approaches are preferred because the classic culture-dependent methods are time-consuming and display only 1–10% of the total microbial diversity [1, 5]. The most widely used marker in studies of prokaryotic diversity is the 16S rRNA gene (small subunit DNA). These methods comprise systematic sequencing of environmental 16S rRNA gene libraries. Automated ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis, denaturant/temperature gradient gel electrophoresis, or single-strand conformation polymorphism (SSCP) [13]. Although molecular approaches are limited by bias in DNA extraction and in polymerase chain reaction (PCR) amplification [8, 20, 24, 32, 33], they allow for the

assessment of the representative diversity that is observed in natural conditions.

The SSCP was first developed by Orita *et al.* [23] to detect DNA polymorphism and point mutations, after which it was adapted to pathogen detection [36] and bacterial community studies [27]. In SSCP, the amplified DNA is denatured and diluted to preclude hybridization of the complementary strains. The single strand will adopt a secondary conformation depending on its sequence; the different conformers are then separated by native electrophoresis on flat polyacrylamide gels followed by silver staining [3, 11, 16, 19] or fluorescence detection [9, 30]. Recently, capillary electrophoresis (CE) coupled to fluorescence detection was used to separate and locate the conformers [6, 7, 12, 17, 41]. The use of CE and fluorescence detection (using a labeled primer) presents many advantages: (1) it is not necessary to isolate the single-stranded DNA [30], (2) it avoids the use of harmful products, (3) it shows high sensitivity, and (4) it is compatible with high-throughput experiments [4]. The use of CE–SSCP for high-throughput analysis of soil still needs some improvement, like the choice of the PCR protocol, sample size, and the influence of the operator. Indeed, the higher sensitivity of CE may result in the emergence of artifacts due to these factors. In this article, we tested the effects of the DNA extraction method on the observed bacterial diversity, and we have optimized several conditions for PCR (extension time, DNA polymerase). The soundness of this protocol was verified with DNA extracted from different solid matrices. Finally, we have mimicked a simple ecosystem by mixing DNA purified from individual cultures of bacterial strains.

Materials and Methods

Bacterial Strains and Soil. Different bacterial strains were used as templates to test CE–SSCP in artificially built ecosystems. *Escherichia coli* S17-1 [29], *Pseudomonas fluorescens* [2], and *Bacillus coagulans* [2] were grown in Luria–Bertani medium (MP Biomedicals, Qbiogene, Inc., Illkirch, France) [25] overnight at 30°C with shaking (200 rpm). *Cupriavidus metallidurans* CH34 [21] was grown in TSM medium supplemented with SL7 solution, 0.8 mM $NiCl_2$, and 1 mM $ZnCl_2$ at 30°C for 48 h [21]. A total number of 10^9 cells of each culture were harvested and washed twice with fresh and sterile water. Pure bacterial DNA from *Sphingomonas* sp. CHY-1 [37], *S. typhimurium* E-10 (NCTC 8391), and *Rhodobacter capsulatus* B10 (ATCC 33303) were provided by J. C. Willison (CEA, Grenoble, France). Soil samples were from Arcy sur Cure's caves (Yonne, France), agricultural soil (Rhône, France), infiltration basin sediments (Chassieu, France), constructed wetland (Ain, France), and alpine grassland soil (Col du Lautaret, Hautes-Alpes, France).

DNA Extraction. Bacterial DNA was extracted using The FastDNA™ Kit (MP Biomedicals, Qbiogene) according to recommendations of the manufacturer. Soil DNA extraction was performed in three different ways. Method A involved extraction of a 250 mg sample using the Power Soil™ extraction kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) according to the instructions of the manufacturer. For method B, a pretreatment of the sample was performed as follows: 250 mg of soil was suspended in 0.3 mL of TE buffer (Tris–HCl 20 mM, EDTA 5 mM, pH 8) containing 20 mg/mL of lysozyme (Euromedex, Mundolsheim, France) and incubated for 1 h at 37°C, followed by the addition of 25 µL of proteinase K (20 mg/mL; Euromedex) and further incubation for 2 h at 56°C. This material was further treated with the Power Soil™ extraction kit. Method C consisted of a classical extraction of 5 g of soil according to the method of Zhou *et al.* [40] with the following modifications: The sample was resuspended in 10 mL TE buffer (100 mM Tris, 100 mM EDTA, 100 mM $NaHPO_4$, pH 8), 0.1 mL proteinase K (20 mg/mL), and 0.18 mL lysozyme (20 mg/mL), and incubated for 30 min at 37°C with strong agitation every 5 min. Then, 3 mL SDS (10%), 4.5 mL NaCl (5 M), and 1.5 mL hexadecylmethylammonium bromide (5% in 1 M NaCl) were added and the samples were incubated for 15 min at 65°C. The resuspended samples were frozen with liquid N_2 and thawed at 65°C three times and then centrifuged at 6000×*g* for 10 min at 4°C. Two successive phenol–chloroform–isoamyl alcohol (24:1:1) extractions were performed by transferring supernatants to new tubes, adding the same volume of phenol–chloroform–isoamyl alcohol and centrifuging for 5 min at 10,000×*g*. The nucleic acids were precipitated by cold ethanol [25] and incubation at −80°C for 15 min. The pellet of crude nucleic acids was obtained by centrifugation at 16,000×*g* for 20 min at room temperature, washed with cold 70% ethanol, dried, and suspended in 0.5 mL of TE buffer. The integrity of the extracted DNA was checked by electrophoresis on a 2% agarose gel 1× TBE (89 mM Tris base, 89 mM borate, 2 mM EDTA). DNA concentration was estimated either by fluorimetry using the PicoGreen® DNA quantification kit (Invitrogen SARL, Molecular Probes, Cergy Pontoise, France) or by comparing the fluorescence intensity in agarose gel electrophoresis with those of DNA Molecular Weight Markers XIII (Roche Molecular Biochemicals, Penzberg, Germany).

PCR Assays. The V3 region of the 16S rRNA gene, corresponding to a 205 bp fragment in *E. coli*, was used as a diversity marker by performing PCR using the

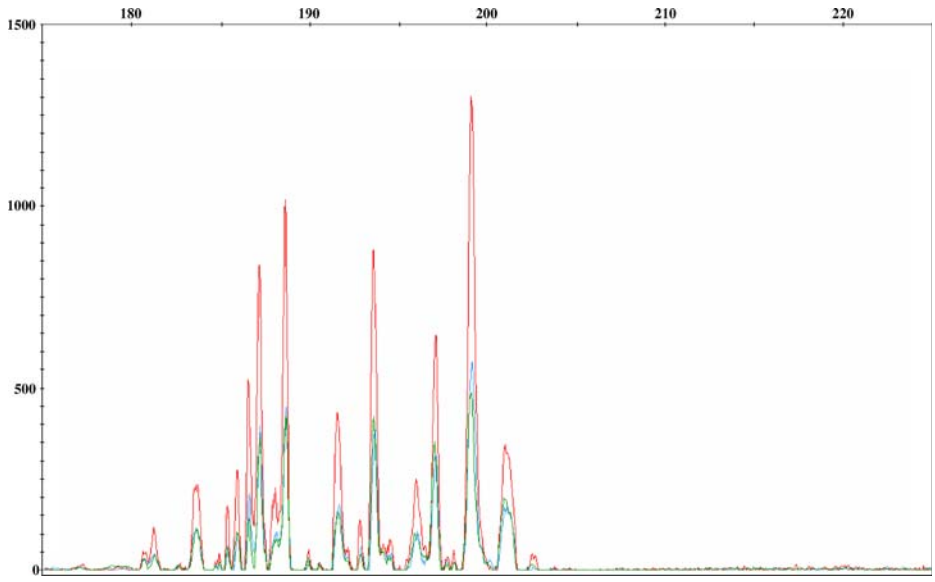

Figure 1. PCR extension time effect on SSCP profiles. Red, 15 s; blue, 30 s, and green 45 s. DNA was extracted from alpine grassland soil using the Power Soil™ extraction kit and amplified by PCR with Isis™ DNA polymerase. *X* axis, molecular weight (bp), which is correlated to migration time; *Y* axis, relative fluorescence intensity. (Color figure online)

primers W49 F (5′-ACGGTCCAGACTCCTACGGG-3′) [6] and W104 R (5′-GTGCCAGCAGCCGCGGTAA-3′) [41]. W104 was labeled with 5′-fluorescein phosphoramidite (FAM). Three different DNA polymerases were tested: Isis™ (MP Biomedicals, Qbiogene), Phusion™, and DyNazyme™ (Finnzymes Oy., Ozyme, Saint Quentin en Yvelines, France). Each PCR mixture contained 0.26 μM of each primer, 0.05 mM of each dNTP, 1× of the furnisher-provided buffer, 0.5 U of DNA polymerase, and 1 μL of environmental DNA (0.02–0.2 ng DNA/μL PCR reaction) in a final volume of 25 μL. Ultrapure water was used for all experiments. The PCRs of cultivated strains were realized with 5 ng of bacterial genomic DNA for each strain and with 0.72 ng (5 ng in total) of strain mix. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France), as recommended by the manufacturer. Three different PCR conditions were tested by changing the extension step: an initial denaturation at 94°C for 2 min followed by 30 cycles of 15 s at 94°C, 15 s at 56°C, 15 s, 30 s, or 45 s at 72°C, and a final extension of 7 min at 72°C. PCR products were visualized on a 1.5% agarose gel. For analysis

Table 1. Diversity observed in SSCP profiles generated by three extension times, three extraction methods, three DNA polymerases, or five environmental samples

Studied factor	*Variables*	*Detected peaks (observed peaks)*	*Shannon index H*
Extension time (s)[a]	15	23 (29)	2.60
	30	17 (26)	2.48
	45	18 (27)	2.49
DNA polymerase	DyNAzyme™[a]	13 (15)	2.30
	Phusion™[a]	14 (27)	2.37
	Isis™[a]	23 (29)	2.60
	DyNAzyme™[b]	5 (9)	0.60
	Phusion™[b]	9 (14)	1.60
	Isis™[b]	10 (14)	1.65
Extraction method[c]	PowerSoil™ kit	18 (24)	2.56
	PowerSoil™ kit + pretreatment	14 (18)	2.23
	Classical method	15 (22)	2.61
Environmental samples	Calcite	10 (14)	1.65
	Infiltration basin	9 (21)	1.73
	Agricultural soil	12 (24)	2.19
	Constructed wetland	18 (24)	2.56
	Alpine grassland soil	18 (29)	2.58

Samples were extracted with the PowerSoil™ kit, except for the extraction method test, and amplified with Isis™, except for in the DNA polymerase test. The peak amount and Shannon–Weaver index were calculated from data given by GeneMapper™ Software.

[a]Alpine grassland soil.

[b]Calcite.

[c]Soil from constructed wetland.

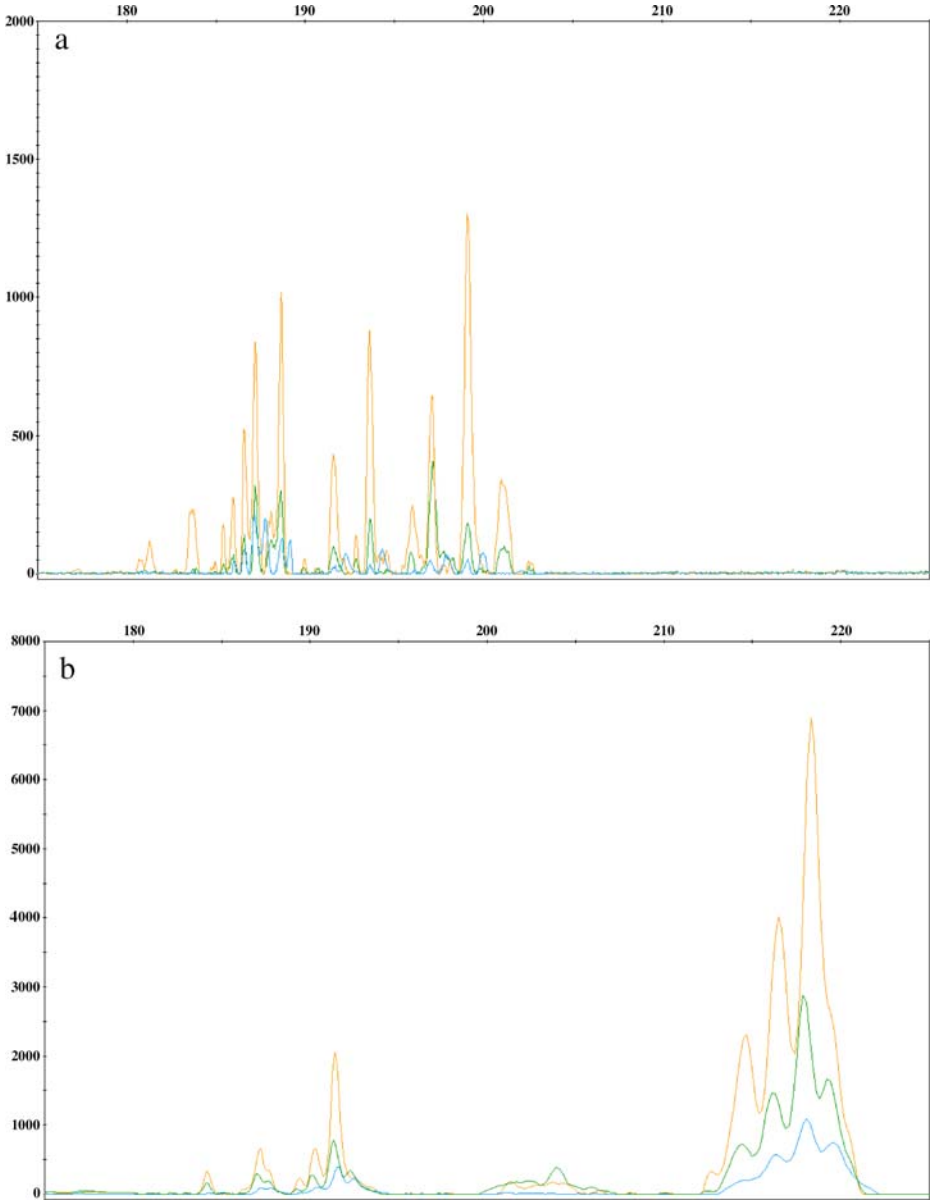

Figure 2. Single-strand conformation polymorphism profiles of (a) alpine grassland soil and (b) cave calcite sediments generated by three different polymerases; orange, Isis™; green, Phusion™ (proofreading polymerases); and blue, Dynazyme™ (mixture of proofreading/*Taq* polymerase). *X* axis, molecular weight (bp); *Y* axis, relative fluorescence intensity. (Color figure online)

of reproducibility, two independent experiments were repeated, such that triplicate PCR amplifications of the same sample were performed by three different operators.

Capillary Electrophoresis Single-Strand Conformation Polymorphism. A 1-μL aliquot of the PCR-SSCP product was mixed with 10 μL of formamide Hi-Di (Applied Biosystems, Courtaboeuf, France) and 0.2 μL of the standard internal DNA molecular weight marker Genescan-400HD ROX (Applied Biosystems). Samples were denatured at 90°C for 2 min and immediately cooled in ice. CE–SSCP was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using a 36 cm length capillary. The nondenaturing polymer consisted of 5% GenScan polymer, 10% glycerol and 3200 Buffer (Applied Biosystems). Running buffer consisted of 10% glycerol and 3200 buffer. The injection time and voltage were set to 22 s and 1 kV, respectively. Electrophoresis was performed at 32°C for 25 min.

SSCP Data Analysis. The peaks of the SSCP profiles were detected with the GeneMapper™ Software version 3.7 (Applied Biosystems), using Genescan-400HD ROX as a size standard, defining an apparent size for each peak. Thus, the location of peaks is arbitrarily expressed in base pairs (bp). For statistical comparison of SSCP profiles, the list of peaks detected by GeneMapper™ and their surfaces were analyzed. Because the data files did not follow a log-normal distribution, we used Spearman's correlation coefficient (S) to compare the SSCP profiles.

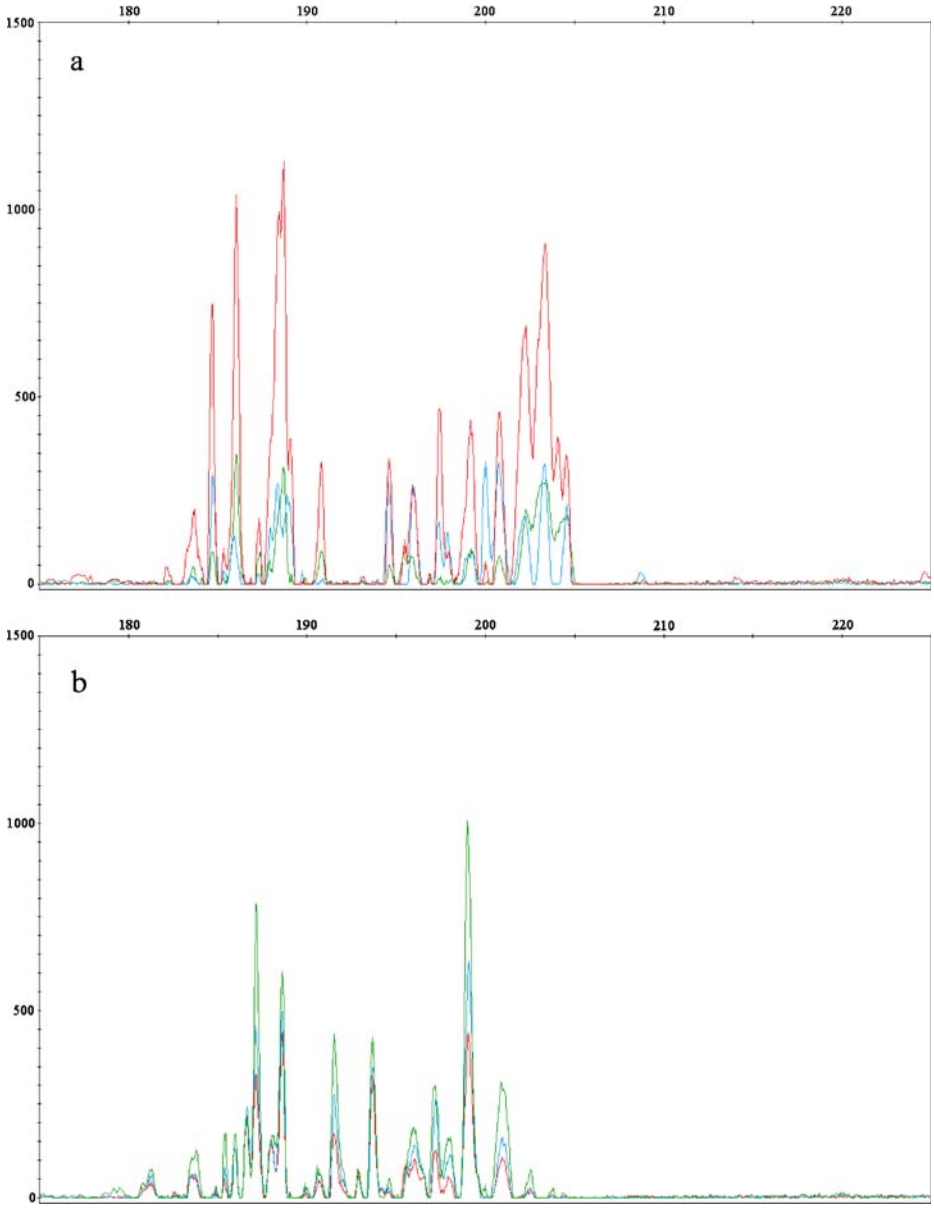

Figure 3. Single-strand conformation polymorphism profiles testing (a) extraction types; blue, classical; red, using the Power Soil™ extraction kit; and green, using the Power Soil™ extraction kit with an initial lysis step; and (b) reproducibility of extractions using the Power Soil™ extraction kit conducted by three different operators. DNA was extracted from alpine grassland soil, and PCR amplification was conducted with Isis™ DNA polymerase. *X* axis, molecular weight (bp); *Y* axis, relative fluorescence intensity. (Color figure online)

The variable of interest was p_i = area of one peak/ total area. The fluorescence intensity levels between SSCP profiles were compared with the Kruskal–Wallis test (K.W), using the data retrieved from GeneMapper™. Statistical analyses were performed with MINITAB™ 13.31 software (Minitab, Ltd., Paris, France) with a confidence interval of 95%. The diversity Shannon index (H) was calculated as $-\Sigma(p_i \ln p_i)$.

Results

Choice of Primers and Template Concentration. Based on previous studies [6, 7, 41], we have used the universal primers, W49 F and W104-FAM R, that amplify the V3 region of the 16S rRNA gene (180–205 bp). To minimize the formation of chimerical amplicons [33], we have used an excess of primers.

These primers generate PCR products (amplicons) expected to migrate between 180 and 205 bp (see Materials and Methods). Additionally, these primers have already been used in bacterial diversity studies. One source of stochastic bias in PCR is the amount of template DNA [24]. Whereas an excess of template DNA may distort the relative representation of certain ribotypes, an insufficient amount of template may preclude the detection of less represented ribotypes and result in low reproducibility. On the other hand, because DNA isolated from soil is often contaminated with DNA polymerase inhibitors [35, 38], it is often necessary to work with a low DNA concentration. We have tested the

Table 2. Capillary electrophoresis single-strand conformation polymorphism reproducibility for prokaryotic diversity studies in alpine grassland soil

Reproducibility test	*Detected peaks (observed peaks)*	*Shannon index H*
Intraoperator extraction	18 (23)	2.576
	20 (23)	2.608
Interoperator extraction	20 (23)	2.608
	18 (23)	2.510
	18 (24)	2.510
Intraoperator PCR	12 (24)	1.939
	14 (24)	1.938
Interoperator PCR	11 (24)	1.834
	14 (24)	1.938
	19 (26)	2.204

Reproducibility tests were conducted twice for extraction. PCRs were carried out in duplicates by different operators. The peak amount and Shannon–Weaver index were calculated from data given by GeneMapper™ software.

effect of template concentrations using DNA isolated from alpine grasslands. The concentrations used were 0.02, 0.2, and 2 ng of template DNA per microliter of PCR reaction. For 0.02 and 0.2 ng/μL of template, the CE–SSCP profiles were similar, but in the case of 2 ng/μL of template, some peaks disappeared, whereas the height of other increased (data not shown). These quantities are in agreement with those previously when using mixes of total DNA isolated from three strains [24]. Thus, 0.02–0.2 ng/μL of DNA was used in the following experiments.

Extension Time Effect. In studies targeting the V3 region, the extension times used varied from 30 s to 1 min. However, during preliminary studies, we observed that a 1-min extension time in PCR led to fragments bigger than 200 bp. In fact, even with a variable polymerization rate for the thermostable-DNA polymerase, the optimum extension time for 200 bp is less than 30 s. We hypothesized that a reduction in the extension time could eliminate the high molecular weight fragment and improve the yield of the 200 bp amplicon. Indeed, the larger fragment was not detected when using shorter extension times. The CE–SSCP patterns (number and area of the peaks) were similar for all the studied extension times, but their fluorescence intensity differed (Fig. 1). The low-intensity peaks were not detected by the GeneMapper™ software (Fig. 1; Table 1). Consequently, statistical analysis showed that the profiles corresponding to 30 and 15 s extension times were correlated ($S = 0.528$; $P = 0.029$), but were not correlated to the one corresponding to 45 s ($S = 0.199$ and 0.091; $P = 0.428$ and 0.767, respectively). The data exported from GeneMapper™ was also used for the Shannon index calculation (Table 1) to determine the effects of the different extension times on diversity. The highest Shannon index value was observed for an extension time of 15 s. Finally, the total amount of fluorescence in the analyzed region was not statistically significant (K.W. = 2.22; $P = 0.330$) for the different elongation times. For the following experiments, we used an extension time of 15 s.

Effect of DNA Polymerase on the SSCP Profile. To assess whether the different thermostable DNA polymerases performed similarly, we tested two different proofreading enzymes with high processivity: Phusion™ and Isis™. We also tested DyNAzyme™, a proprietary mixture of a proofreading and a Taq-like polymerase. We used DNA isolated from two different ecosystems as a template: the first was from a low-diversity calcite sample [2] and the second was from an alpine grassland soil that displayed more complex patterns. Visual analysis of the electropherograms suggests that the total fluorescence intensity is higher for samples produced by Isis™ (Fig. 2a and b). This difference is statistically supported for alpine grassland soil samples (K.W. = 5.25; $P = 0.072$; Fig. 2a), but not for the calcite samples (K.W. = 2.26; $P = 0.322$; Fig. 2b).

The analysis using GeneMapper™ revealed that the number of peaks detected varied according to the enzyme used (Table 1). For the calcite samples, the shape of the profiles is similar for Phusion™ and the other polymerases ($S = 0.994$; $P = 0.006$ with DyNAzyme™; $S = 0.933$ $P < 0.001$ with Isis™), but a difference was observed between Isis™ and DyNAzyme™ ($S = 0.930$; $P = 0.07$). However, GeneMapper™ detected different number of peaks for each of the three enzymes used (Table 1; Fig. 2b). For the alpine soil, there is no significant difference between the profiles obtained with the two proofreading polymerases ($S = 0.564$; $P = 0.036$), but the profile obtained with DyNAzyme™ was found to be different ($S = 0.073$; $P = 0.852$ with Isis™; $S = 0.399$; $P = 0.328$ with Phusion™), both in the number of peaks and the relative abundance of each peak (Table 1, Fig. 2a). It is worth noting that, for both samples, the peaks obtained with DyNAzyme™ were shifted when compared with those of the proofreading enzymes. The failure to detect certain peaks was reflected in the Shannon index (Table 1). These results indicate that Isis™ is the best-suited enzyme for microbial diversity studies, probably due to the high fluorescence recovery. We have used this enzyme in the following experiments.

Influence of DNA Extraction Method. Because some bacteria may resist specific extraction treatments, we have tested three different extraction methods (see Materials and Methods) to extract DNA from the soil of a constructed wetland.

The number of peaks and the diversity index vary with the extraction method. Method A displays the highest diversity (Table 1). The CE–SSCP profiles are shown in Fig. 3a. Concerning fluorescence intensity, Kruskal–Wallis test revealed significant differences between the profiles generated by the three methods

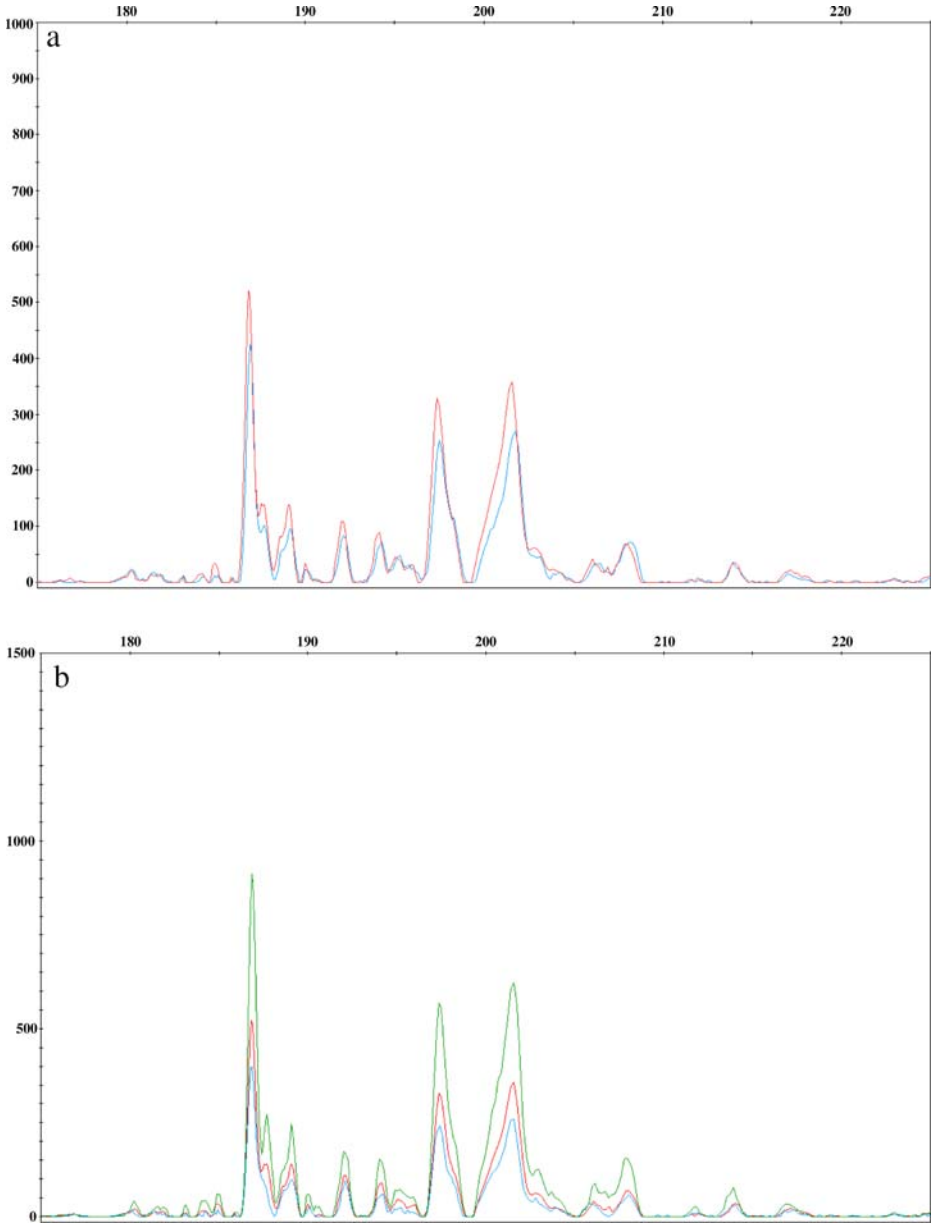

Figure 4. Single-strand conformation polymorphism profiles showing (a) reproducibility for one operator and (b) reproducibility between the three operators. DNA was extracted from alpine grassland soil using the Power Soil™ extraction kit and PCR was conducted with Isis™ DNA polymerase. *X* axis, molecular weight (bp); *Y* axis, relative fluorescence intensity. (Color figure online)

(K.W. = 17.25; $P < 0.01$). The relative abundance of the predominant ribotype was very different for method C ($S = 0.270$; $P = 0.373$ with A; $S = 0.207$; $P = 0.518$ with B), whereas samples obtained with methods A and B showed only a weak difference ($S = 0.569$; $P = 0.054$). These results support the use of the commercial kit for DNA extraction.

Extraction and PCR Reproducibility. Bacterial distribution heterogeneity may display a bias when using 250 mg of soil [13]. DNA extraction may also be biased by the operator (i.e., when weighing the soil). To assess these potential biases, three different extraction operators conducted extractions in duplicate. The results are shown in Fig. 3b and Table 2. No significant differences were observed between the duplicates of one operator, or between the three operators ($S > 0.623$; $P < 0.01$ between all profiles).

To assess the stochastic biases of the PCR and the influence of operators, reproducibility tests were conducted twice by three different PCR operators. We did not observe any significant difference neither in the replica of one operator (Fig. 4a) nor between the three operators (Fig. 4b; $S > 0.863$; $P < 0.006$ between all profiles; Table 2).

Analysis of Environmental Samples. Different soil types were studied here to test the effect of soil complexity

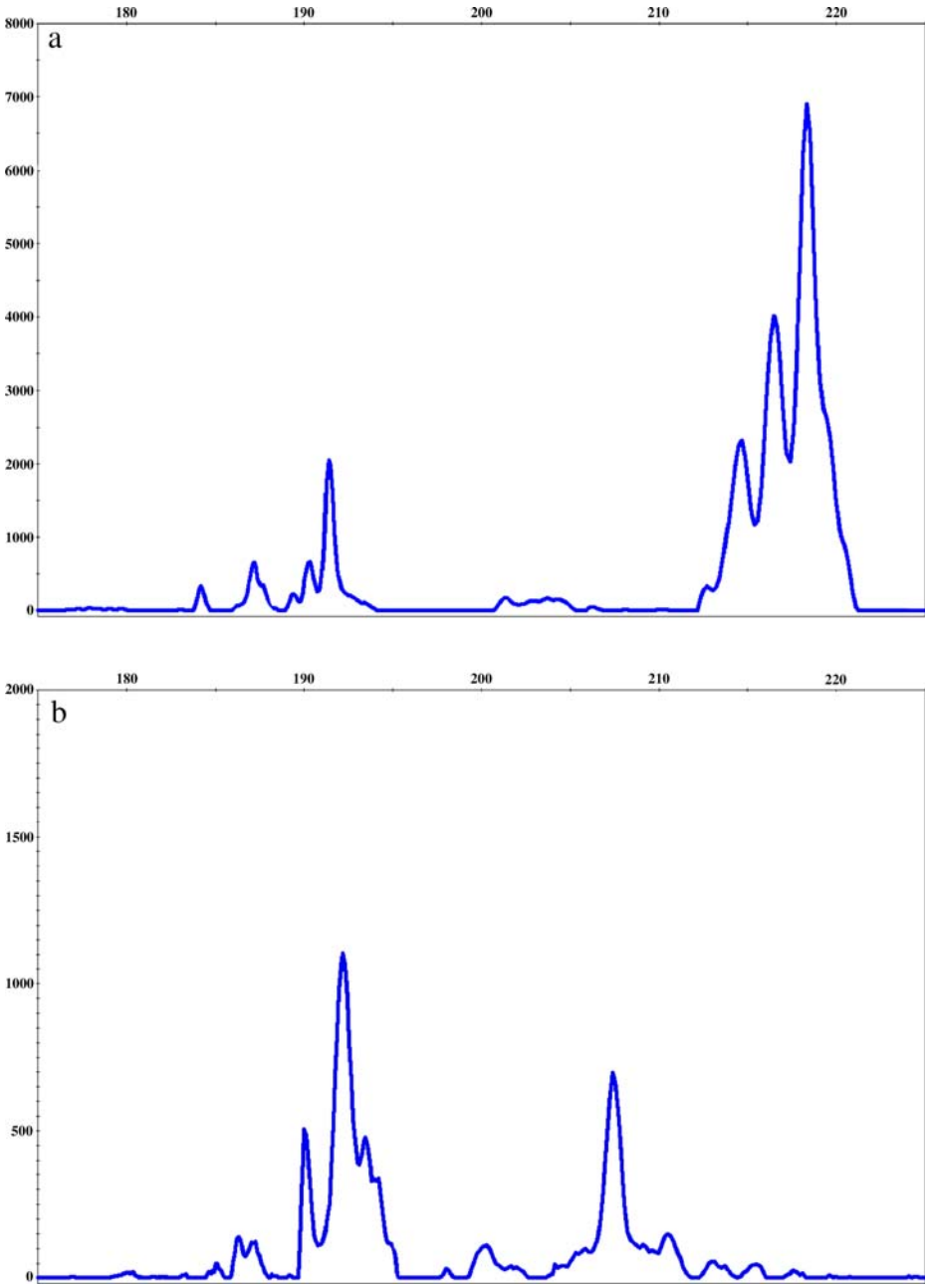

Figure 5. Single-strand conformation polymorphism profiles of (a) cave calcite sediments; (b) infiltration basin polluted sediments; (c) agricultural soil; (d) macrophyte bed soil treating waste water; (e) alpine grassland soil. Extractions were performed with the Power Soil™ extraction kit and PCR amplification was conducted with Isis™ DNA polymerase. *X* axis, molecular weight (bp); *Y* axis, relative fluorescence intensity.

on the resulting SSCP profiles and, thus, to check the coherence of SSCP results (Fig. 5). The soils were expected to exhibit different bacterial diversity. The calcite sediments come from a karstic cave [2] and contain little organic matter; accordingly, the SSCP profile presented only a few peaks (Fig. 5a).

We have also analyzed infiltration basin sediments containing around of 10^9 CFU/g DW of cultivatable bacteria. These sediments contain complex organic molecules that may inhibit thermostable DNA polymerases. Indeed, the yield of the PCR reaction was much lower than that for the other samples, suggesting that the extraction procedure did not completely remove the inhibitors. Although the SSCP profile obtained with this sample is more complex than the previous one (Fig. 5b), the data analysis software failed, once again, to detect many peaks.

When using other samples from more complex ecosystems (agricultural soil, soil from a constructed wetland, and alpine grass soil), the CE–SSCP profiles displayed more peaks that were readily detected by the software (Fig. 5c–e; Table 1). The statistical analysis of the CE–SSCP profiles indicates that they were significantly different ($S < 0.465$; $P > 0.176$ between all profiles). Thus, the data obtained using the PCR and CE–SSCP

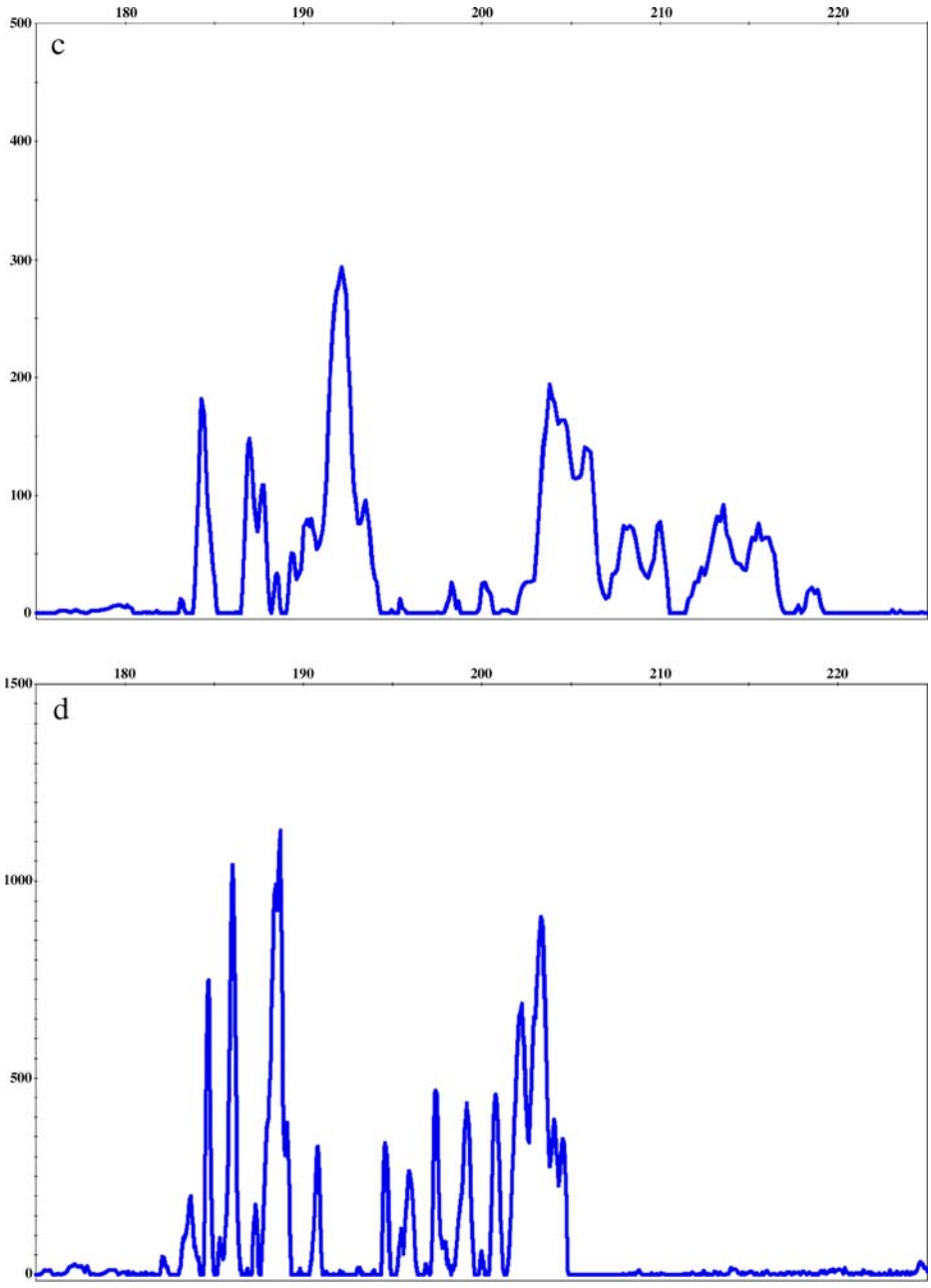

Figure 5. (continued).

conditions described here is indicative of the microbial diversity of a given ecosystem.

Mimicking a Simple Ecosystem with DNA from Cultured Strains. To assess if the profiles obtained for the environmental samples were representative of the ribotypes present in the samples, we tested the PCR and CE–SSCP procedures using genomic DNA extracted from pure cultures. We used seven bacterial strains, including two isolated from the calcite samples (Table 3).

DNA from each individual strain or a DNA mixture was amplified using the primers FAM-W104 F and HEX-W49 R. The use of two fluorescent labels allowed us to assess the behavior of each strand from the V3 region during native CE. The CE–SSCP profiles are shown in Fig. 6. The reverse strand (FAM-label) migrates faster than the forward strand (HEX-label). This difference in migration has already been observed with clinical isolates of *Mycobacterium* [9]. Swapping of the fluorescent label (HEX-W104 and FAM-W49) slightly changed the apparent size of the peak (±1 base), but did not change this pattern nor the shape of the peaks (see below; data not shown). It did, however, significantly affect the migration of the strands. Surprisingly, when analyzing DNA from individual pure cultures, we found more than one peak for an isolated bacterial strain (Fig. 6). The apparent

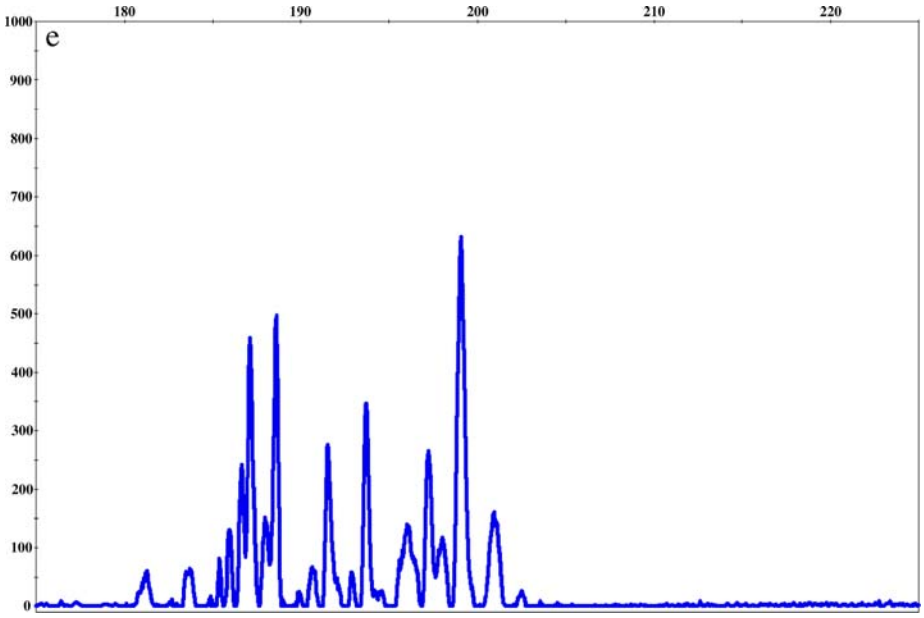

Figure 5. (continued).

molecular size variance of the main peaks between different electrophoreses was found to be ±1 base (Table 3).

For the reverse strand, a major peak preceded by one or two minor (satellite) peaks was observed systematically (Fig. 6). The peaks corresponding to *P. fluorescens*, *B. coagulans*, and *C metallidurans* CH34 migrate to virtually the same position, preventing discrimination of the mixture components. Regarding *R. capsulatus* and *Sphingomonas* sp., the main peaks migrate to very close points (one apparent base), and the satellite peak of the latter migrates near the main peak of the former (<0.5 apparent base). In the mixture, slight differences in the migration of such satellite peaks (<1 apparent base) may result in the formation of shoulders rather than two well-resolved peaks. If attribution of ribotypes in the mixture is also based on the shape of the peaks, it may lead to erroneous interpretations. Finally, *Salmonella thyphimurium* and *E. coli* are well resolved in the mixture. When considering the forward strand, for most of the strains (excluding *P. fluorescens*), two peaks of comparable fluorescence were systematically observed (Fig. 6). In the case of *P. fluorescens*, three peaks were systematically observed, but their relative size varied among experiments. The forward-strand conformers were better separated that those of the reverse strand. In fact, if we consider only the peaks showing the highest apparent size, the individual strains are all resolved by at least 1 apparent base. Again, using the data from the individual strains, it is possible to interpret the mixture. However, the complexity of the sample is usually unknown; thus, such a profile may lead to errors when estimating the species number. The presence of the multiple peaks per ribotypes may have an impact on the ribotype number or diversity index estimation.

Discussion

The development and improvement of techniques allowing an estimation of the bacterial phylotypes are important for studying the dynamics of the microbial compartment in a given ecosystem (i.e., seasonal dynamics, impact of pollutants, or human activity, etc.). The most commonly used methods are ARISA and T-RFLP, which have also been adapted for flat-gel sequencing machines [10, 13]. CE–SSCP also allows for high-throughput analysis [4]. In SSCP, the optimal size of the PCR product is about 150–250 bp. In fact, fragments too long in size are conducive to hidden variability because of their three-dimensional conformations [14, 15, 28, 31]. A 150–250 bp fragment is convenient because the hypothetical risks of PCR bias linked to the amplicon size are reduced; it allows the use of fragmented DNA as a

Table 3. Real size and CE–SSCP electrophoretic mobility values in bp of each strand of the V3-16S RNA gene

Bacterial strain	*Real size*	*W49-HEX strand*	*W104-FAM strand*
P. fluorescens	205	215.42±0.42	218.42±0.33
B. coagulans	205	208.14±0.25	217.69±0.33
C. metallidurans CH34	205	205.72±0.19	217.44±0.20
E. coli S17-1	205	194.85±0.20	207.62±0.21
Sphingomonas sp.	180	186.82±0.21	192.05±0.23
S. typhimurium	205	191.64±0.32	201.97±0.14
R. capsulatus	180	185.78±0.22	190.69±0.31

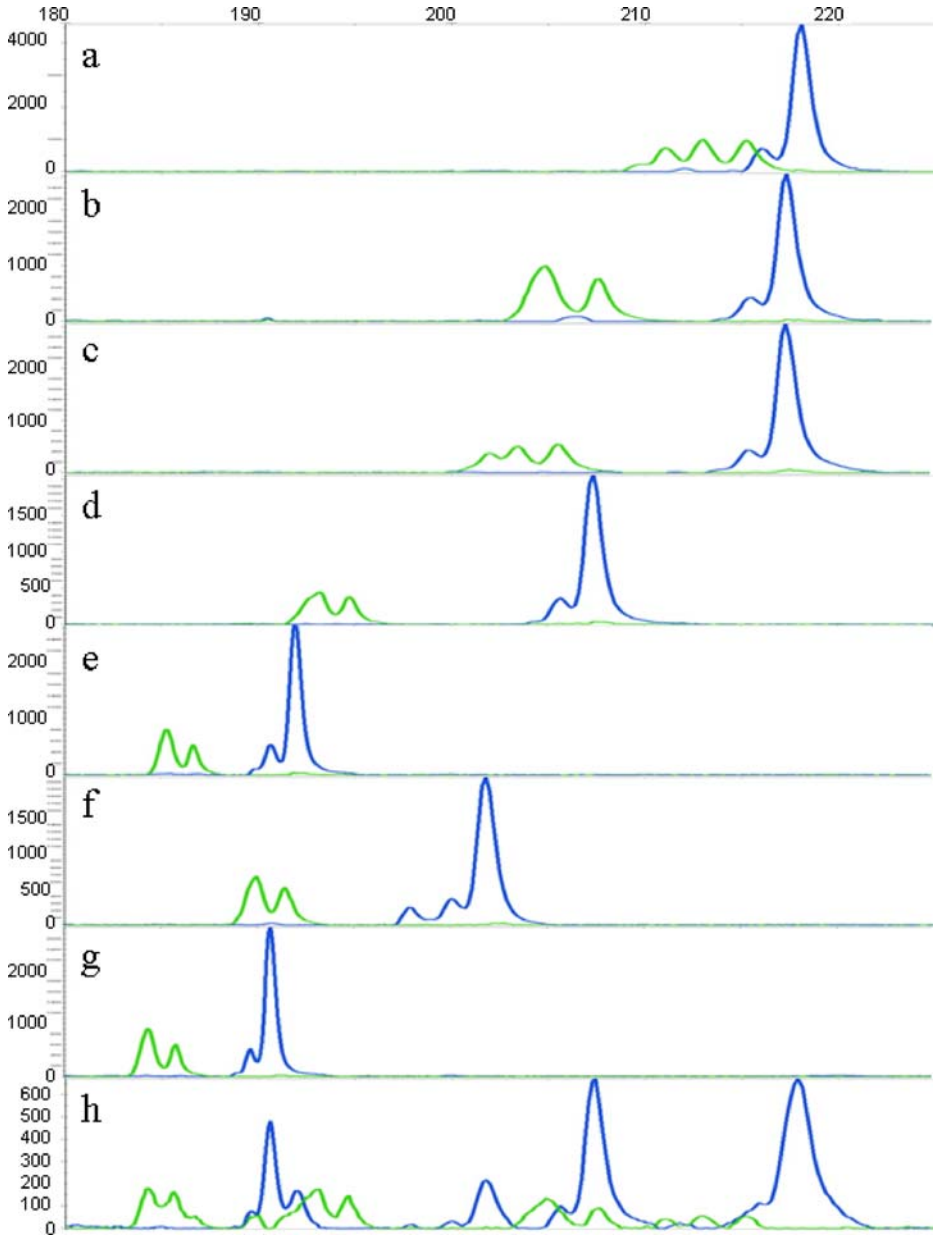

Figure 6. Capillary electrophoresis single-strand conformation polymorphism peak of PCR-amplified 16S DNA from pure-culture bacteria, using HEX-W49 F (*green*) and FAM-W104 R (*blue*) primers. Bacterial peaks (*open green peaks*) were assigned a mobility value in bp relative to an internal standard (not shown). (a) *P. fluorescens*; (b) *B. coagulans*; (c) *C metallidurans* CH34; (d) *E. coli* S17-1; (e) *Sphingomonas* sp. CHY1; (f) *S. typhimurium*; (g) *R. capsulatus*; (h) mixture of all bacteria in equal amounts before PCR. (Color figure online)

template, it reduces the impact of DNA polymerase inhibitors, and also reduces the impact of DNA polymerase errors. Previous studies have shown that region V3 is the most informative region of DNA [6, 12, 26]. In this study, we report on the suitability of CE–SSCP for high-throughput studies of environmental microbiology. The major concerns were the reproducibility and the consistency of results. To our knowledge, this is the first comprehensive study of the suitability of CE–SSCP for high-throughput microbial diversity studies.

It is worth stressing that the analysis of SSCP results cannot be fully exploited due to the limitations of the GeneMapper™ Software. As mention before, the observed peaks (Figs. 4a–b; Table 2) are different from the detected peaks (Table 2). The undetected peaks have a low intensity. Thus, their influence on the correlation analysis is only marginal. In addition, the signal/noise ratio assessment is different for each sample, preventing the use of a constant threshold in GeneMapper. Moreover, the analysis software does not always take the shoulders into account, which can correspond to one or several ribotypes. All of these limitations lead to an underestimation of diversity and may explain some of the observed differences. Effects of software limitations are thus reflected by changeable Shannon indices for the same soil treated in the same way, but not in the statistical analysis. Furthermore, GeneMapper™, previously developed for microsatellite and amplified fragment length polymor-

phism studies, automatically fails to detect several standard peaks, requiring that each run should be manually corrected.

While using mixtures of pure DNA from three bacteria, Polz *et al.* [24] found that the optimum template concentration was 0.2 ng/μL of PCR reaction. Using an environmentally complex sample, we have found the same optimal concentration. Given that total soil DNA includes DNA from fungi, plant, and small insects, this result is unexpected.

The main issue for the choice of the DNA polymerase is that mutations occurring in the first few cycles of the PCR reaction may introduce an artificial distortion of microbial diversity in a given sample. For this reason, proofreading DNA polymerases may be preferred to Taq-like DNA polymerases. Indeed, most studies using CE–SSCP use proofreading polymerases, but there is no consensus for the type of enzyme that should be used in this analysis [6, 41]. Indeed, the use of Taq polymerase enzymes has recently been reported [12, 30]. Our results also show that the yield of the PCR reaction favors the use of proofreading DNA polymerases.

We have found that the number of peaks and their relative fluorescence intensity varied with the DNA extraction method. However, given that the actual ribotypes contained in soil were unknown, it is not possible to identify the most realistic method. Three factors prompted us to choose the PowerSoil™ kit for DNA extraction. First, most of the peaks were present with samples prepared using this kit. Second, it is more suitable than the procedure described by Zhou *et al.* [40] for high-throughput studies. Finally, the reproducibility was independent of the operator. We have obtained clearly different SSCP profiles for ecosystems from different solid matrixes. The similar migration of DNA isolated from different strains was observed here and elsewhere [12]. In environmental samples, one band can represent more than one ribotype [22]. Direct cloning of short DNA fragments isolated from flat gels may give biased results. In CE–SSCP, where the DNA of the peak cannot be isolated, an indirect strategy can be used to amplify and clone the complete 16S rRNA gene. The recombinant plasmids can then be submitted to sequencing and CE–SSCP. Random sequencing experiments may also be useful for validation of the diversity index calculation using the CE–SSCP peaks. Because one ribotype may be represented by more than one peak and each peak may represent more than one ribotype, the calculation of diversity indices is not straightforward and needs further theoretical study.

The CE–SSCP analysis of DNA from cultured bacterial strains was particularly informative. In most cases, we observed the presence of multiple peaks, whose relative size was dependent on the primer. This phenomenon was previously observed when studying 16S rRNA gene plasmid clones from Chrenoarcheotes [30]; it was suggested to be due to the presence of different conformers. In our case, because we have used total DNA, it may be argued that the different peaks represent the different 16S rRNA gene operons present in each strain. However, this was not the case for *P. fluorescens* and *C. metallidurans* whose genome sequences are available (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Present work in our laboratory suggests that multiple peaks for a single strain result from PCR artifacts rather than from different conformers (J. Gury *et al.*, in preparation). By using a fluorescent label in each primer, we confirmed that the conformation information is different for each strand. In the few experiments performed here, the forward strand is more informative; however, in other species, it maybe the converse. Thus, the use of double labeling is recommended.

The information gained from SSCP patterns in terms of species richness or diversity index is not straightforward. First, a single ribotype may display two or more peaks (Fig. 6). Second, one peak can correspond to many ribotypes [13, 26]. Thus, it is difficult to assess the influence of these biases on the final profile. However, recent work indicated the shape of the profiles is more informative than the number of peaks [18]. Therefore, variations on peak number may not affect the diversity index estimation. Further modeling work is needed to solve this problem.

The procedure outlined here can be performed extremely quickly. Using a 96-well plate, DNA extraction can be performed in one day; DNA quantification, PCR (2 hours), and agarose gel electrophoresis can be performed on a second day, and the CE takes 3 h in a 16-capillar analyzer. The bottleneck of the whole procedure is the analysis of the sequences, owing to the unsuitability of the software to work in size mode. However, analysis using "data point" is a faster alternative. In conclusion, the PCR-CE–SSCP method described here is promising for high-throughput microbial ecology studies, for instance spatiotemporal dynamics or biogeography studies. CE–SSCP, in a relatively quick and simple way, can produce a snapshot of the bacterial communities of an environmental sample. The comparison of similar ecosystems under different conditions can be performed in a relatively short time, allowing extensive studies on the spatial distribution, seasonal fluctuations, and dynamic follow-up of prokaryotic soil diversity.

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Chapitre I – Article B

CE-SSCP and CE-FLA, simple and high throughput alternatives for fungal diversity studies

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CE-SSCP and CE-FLA, simple and high-throughput alternatives for fungal diversity studies

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Abstract

Fungal communities are key components of soil, but the study of their ecological significance is limited by a lack of appropriated methods. For instance, the assessment of fungi occurrence and spatio-temporal variation in soil requires the analysis of a large number of samples. The molecular signature methods provide a useful tool to monitor these microbial communities and can be easily adapted to capillary electrophoresis (CE) allowing high-throughput studies. Here we assess the suitability of CE-FLA (Fragment Length Polymorphism, denaturing conditions) and CE-SSCP (Single-Stranded Conformation Polymorphism, native conditions) applied to environmental studies since they require a short molecular marker and no post-PCR treatments. We amplified the ITS1 region from 22 fungal strains isolated from an alpine ecosystem and from total genomic DNA of alpine and infiltration basin soils. The CE-FLA and CE-SSCP separated 17 and 15 peaks respectively from a mixture of 19 strains. For the alpine soil-metagenomic DNA, the FLA displayed more peaks than the SSCP and the converse result was found for infiltration basin sediments. We concluded that CE-FLA and CE-SSCP of ITS1 region provided complementary information. In order to improve CE-SSCP sensitivity, we tested its resolution according to migration temperature and found 32 °C to be optimal. Because of their simplicity, quickness and reproducibility, we found that these two methods were promising for high-throughput studies of soil fungal communities.

Keywords: Biodiversity; Fungi; Soil; High-throughput; CE-FLA; CE-SSCP

1. Introduction

The composition and dynamic of the microbial community play a key role in the soil functioning. In this context, fungi represents a large (if not predominant) amount of soil biota (Ananyeva et al., 2006) and hold a central place in the subterranian food web since they are able to decompose organic matter with extra-cellular enzymes and allow the relocation of nutrients in plants through mycorrhiza. These fungal associations may be mutualistic or pathogenic, thus influencing the productivity and the colonization ability of plant communities (De Boer et al., 2005; Torsvik and Ovreas, 2002; Wardle et al., 2004; Wardle, 2006). Moreover, biomass, phylogenetic structure and metabolic properties of fungal communities appear to be modulated by environmental conditions (Artz et al., 2007; Gomes et al., 2003; Schadt et al., 2003; Van der Wal et al., 2006). Therefore, the assessment of fungal communities and their role requires a large sample analysis and thus, the use of high-throughput technologies.

Currently, soil microbial communities are monitored by molecular profiling methods, which provide a snapshot of microbial diversity and are a useful preliminary step before deeper analysis by cloning/sequencing (Anderson and Cairney, 2004; Kirk et al., 2004). These methods use total soil DNA as a template for the PCR amplification of marker genes (*i.e.* rRNA genes) using kingdom-specific fluorescent primers (Amann et al., 1995; Anderson and Cairney, 2004; Kumar and Shukla, 2005; White et al., 1990). Fungal specific primers have been designed on 28S, 18S and Internal Transcribed Spacers (ITS) (Anderson et al., 2003a; Anderson and Cairney, 2004; Egger, 1995; White et al., 1990) and are currently used for phylogenetic,

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environmental and biodiversity studies (James et al., 2006; O'Brien et al., 2005; Schadt et al., 2003). Phylotypic diversity of a given sample is assessed through three kinds of methods relying on electrophoresis. The first kind exploits the size variation of a marker gene that is consequently separated according to its length (Fragment Length Analysis (FLA), Automated Ribosomal Intergenic Spacer Analysis (ARISA)). The second type of methods separates the amplicons by their sequences and thus, their conformations. Electrophoresis may be denaturant as for Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE), or native for Single-Stranded Conformation Polymorphism (SSCP). Finally, the third technique relies on both previous methods: PCR products are digested according to their sequence with restriction enzyme, and then separated by their length (Restriction Fragment Length Polymorphism (RFLP) and Terminal-RFLP (T-RFLP)). At this time, T-RFLP and DGGE are widely used for fungal community studies (Anderson et al., 2003b; Arenz et al., 2006; Avis et al., 2006; Dickie and FitzJohn, 2007; Kennedy et al., 2005; van Elsas et al., 2000).

Fungal (and in general microbial) community studies require high-throughput technologies which imply a reduction of manipulation and analyses steps, high reproducibility, and multiplexing possibilities with the concomitant reduction of study time and costs. All the methods described such as ARISA and DGGE are most often performed in flat-gel, which is therefore accessible for most of labs, but does not allow the simultaneous analysis of a large number of samples (Anderson et al., 2003b; Gomes et al., 2003; Hansgate et al., 2005; Ranjard et al., 2001). Capillary Electrophoresis (CE) provides the possibility of analysing a large number of samples in a few hours and has already been coupled to T-RFLP for soil fungal community studies (Klamer et al., 2002; Schwarzenbach et al., 2007). While the FLA and SSCP using CE have been used for studying pathogenic fungi (Chaturvedi et al., 2005; De Baere et al., 2002; Kumar et al., 2005; Kumar and Shukla, 2005; Turenne et al., 1999) and yeast communities in the cheese industry (Callon et al., 2006), they have never been applied to fungi from soil samples.

In this article, we have focused on FLA (denaturing conditions) and SSCP (native conditions) because they allow the analysis of PCR product without any further treatment. Instead of using the complete ITS region, we have selected the ITS1 since CE-SSCP was shown to be more efficient for short DNA fragments (Sheffield et al., 1993). Several studies have shown a weak size polymorphism of ITS2 at the genus level (Kumar and Shukla, 2005; Turenne et al., 1999), and the recent description of a class I intron in ITS1 points out the potential high polymorphism of this sub-region (Egger et al., 1995; Martin and Rygiewicz, 2005). The primers targeting this region were previously tested for their fungal specificity (Kumar et al., 2005; Kumar and Shukla, 2005; Kumar and Shukla, 2006). We compared the CE-FLA end CE-SSCP for their sensitivity, because amplicons of the same size in FLA, which could differ in their sequence, should be separated in SSCP. For this, we used 22 fungal strains isolated from alpine grassland soil. Furthermore, we tested migration temperature effect on CE-SSCP which is strongly linked to the fragment conformation to optimize this technique. Finally, we validated this fingerprinting method by characterizing the molecular signature of fungal communities of an alpine soil and infiltration basin sediments.

2. Material and methods

2.1. Strain isolation, DNA extraction and fungal identification

The 22 fungal strains are listed in Table 1. They were isolated from an alpine grassland soil (massif du grand Galibier, Hautes Alpes, France) as follows: 2 g of soil were suspended in 100 ml SDS 0.05% and agitated for 1 h at room temperature. The suspension was then filtered with Millipore™ membranes (∅80 and 180 μm) and the three fractions were cultured at 25 and 5 °C with an agar alpine medium. This medium was prepared as follow: 400 g of soil were boiled in 1000 ml of water for 1 h. After filtration, 15 g of agar were added and the pH was adjusted for 6.8–7.

A boiling-freezing protocol was used for DNA extraction from isolated fungal strains. Approximately 250 mg of each strain were suspended in 200 μl bi-distilled water and heated for 10 min at 100 °C. Each strain was extracted at least twice. Before adding the DNA extract to the PCR mix, the samples were briefly centrifuged to pellet any cell.

Table 1
Mobility values of the 22 fungi strains in capillary electrophoresis in denaturing condition (FLA) and non-denaturing conditions (SSCP)

Strain name	Strain number	CE FLA mobility value (b)	CE-SSCP mobility value (b)
ND. (Sarcosomataceae)	A68[2]	230.87±0.134	240.60[α]±0.189
Neonectria radicicola	A72[2]	232.75[β]±0.304	238.17±0.189
Cylindrocarpon didymum	A25[2]	233.37[β]±0.115	240.29[α]±0.313
Ampelomyces quercinus	B88[2]	236.08±0.092	245.74±0.131
Preussia intermedia	B4[2]	239.28±0.091	248.50±0.422
Fusarium sp.	B29[1]	ND.	249.17±0.384
Cladosporium sp.	B14[1]	ND.	257.54±0.546
Leptosphaeria bicolor	A81[2]	248.61±0.049	268.35±0.525
Fusarium tricinctum	B24[1,2]	250.02±0.046	252.60±0.390
Cladosporium sp.	B5[2]	253.04±0.038	250.54±0.199 254.24±0.572
ND. (*Epacris microphylla* root associated fungus)	B33[2]	257.50[β]±0.082	286.10±0.171
Toplypocladium inflatum	B65[2]	258.18[β]±0.164	282.44[α]±0.515
Cryptococcus terricola	B52[2]	263.63±0.027	304.26±0.220
Penicillium sp.	A13[2]	266.80±0.112	283.28[α]±0.524
Penicillium sp.	A22[2]	267.72±0.156	282.78[α]±0.132
Pseudogymnoascus roseus	B85[2]	270.22±0.066	276.76±1.019
ND. (*Epacris microphylla* root associated fungus)	B55[2]	272.17±0.078	287.77±0.134
Alternaria alternata	B19[1]	ND.	285.60±0.384
Verticillium suchlasporium	B143[2]	278.76±0.033	301.19±0.556
Mucor hiemalis	B10[1,2]	325.39±0.119	374.62±0.899
Drechslera dematioidea	A31[2]	333.89±0.047	344.94±0.889
Phialocephala sphaeroides	B130[2]	567.49±0.937	617.57±2.085

Mean and SD were calculated with 4 CE-runs, from two different PCR reactions. [1]=strains used in mixture 5, [2]=strains used in mixture of 19 strains. [α]=CE-SSCP co-migrated peaks sample, [β]=CE-FLA co-migrated peaks in fungi mixed sample. ND: not determined.

Each strain was identified by microscopic observation and sequencing of the internal transcribed spacer with ITS5 (see below) and ITS4 (White et al., 1990) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) after sequence correction with SeqScape v2.5 (Applied Biosystems, Courtaboeuf, France).

2.2. Soil DNA extraction

Alpine soil samples came from massif du grand Galibier as cited above. Sediments were sampled in an infiltration basin (Chassieu, France). Soil DNA extractions were performed with the Power Soil™ Extraction Kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) from 250 mg (fresh weight) soil per sample according to the manufacturer's instructions.

2.3. PCR amplification

The ITS1 region of ribosomal RNA genes was amplified at least two times for each strain with the primers ITS5 (5′-GGAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990). The ITS2 primer was labelled in 5′ with fluorescein phosphoramidite (FAM). PCR amplification was performed in a 50 µl final volume consisting of 2.5 mM of $MgCl_2$, 1X of AmpliTaq Gold™ buffer, 20 g L^{-1} of Bovine Serum Albumin, 0.1 mM of each

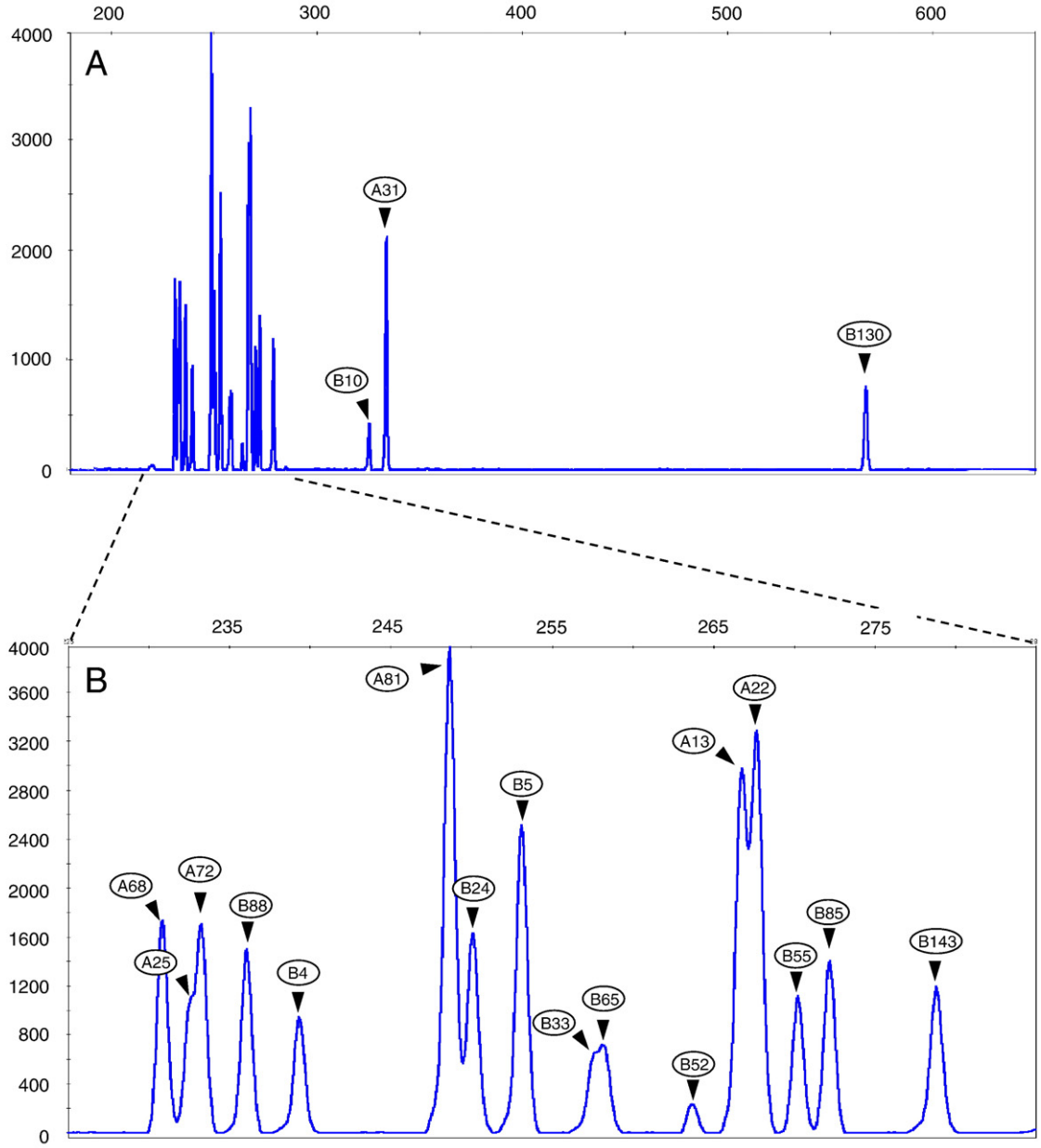

Fig. 1. Capillary electrophoresis profile of ITS1-PCR fragments from 19 fungi strains (see Table 1) in denaturing condition (CE-FLA). A: total fingerprint; B: up-sizing to 225–285 bp. The *y*-axis is relative fluorescence units (rfu), the *x*-axis is size represented in bases.

dNTP, 0.26 μM of each primer, 2 U of DNA polymerase (Applied Biosystems, Courtaboeuf, France) and 2 μl of DNA template. Both isolated strains (Table 1) and soil DNA were used as template. The thermal cycling was carried out according the following protocol: enzyme activation at 95 °C for 10 min; 31 cycles of denaturation at 95 °C for 30 s, hybridization at 56 °C for 15 s, extension at 72 °C for 15 s; and final extension at 72 °C for 7 min. PCR products were visualized on a 2% TBE agarose gel, allowing the DNA concentration assessment, and purified with the Qiaquick™ PCR Purification Kit (Qiagen, Courtaboeuf, France). All strain mixes were obtained by pooling the same concentration (about 1 ng/μl) of each strain ITS1-PCR products. The composition of the mixes is given in Table 1.

2.4. Capillary electrophoreses

Both CE-FLA and CE-SSCP were performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France) in a 36-cm length capillary.

2.5. CE-FLA

In order to check the actual size and quality of ITS1-PCR products, capillary electrophoreses under denaturing conditions were performed using 1 μl of 50-fold diluted PCR products, 10 μl Hi-Di formamide and 0.1 μl internal DNA molecular marker Genescan-500 ROX (Applied Biosystems, Courtaboeuf, France).

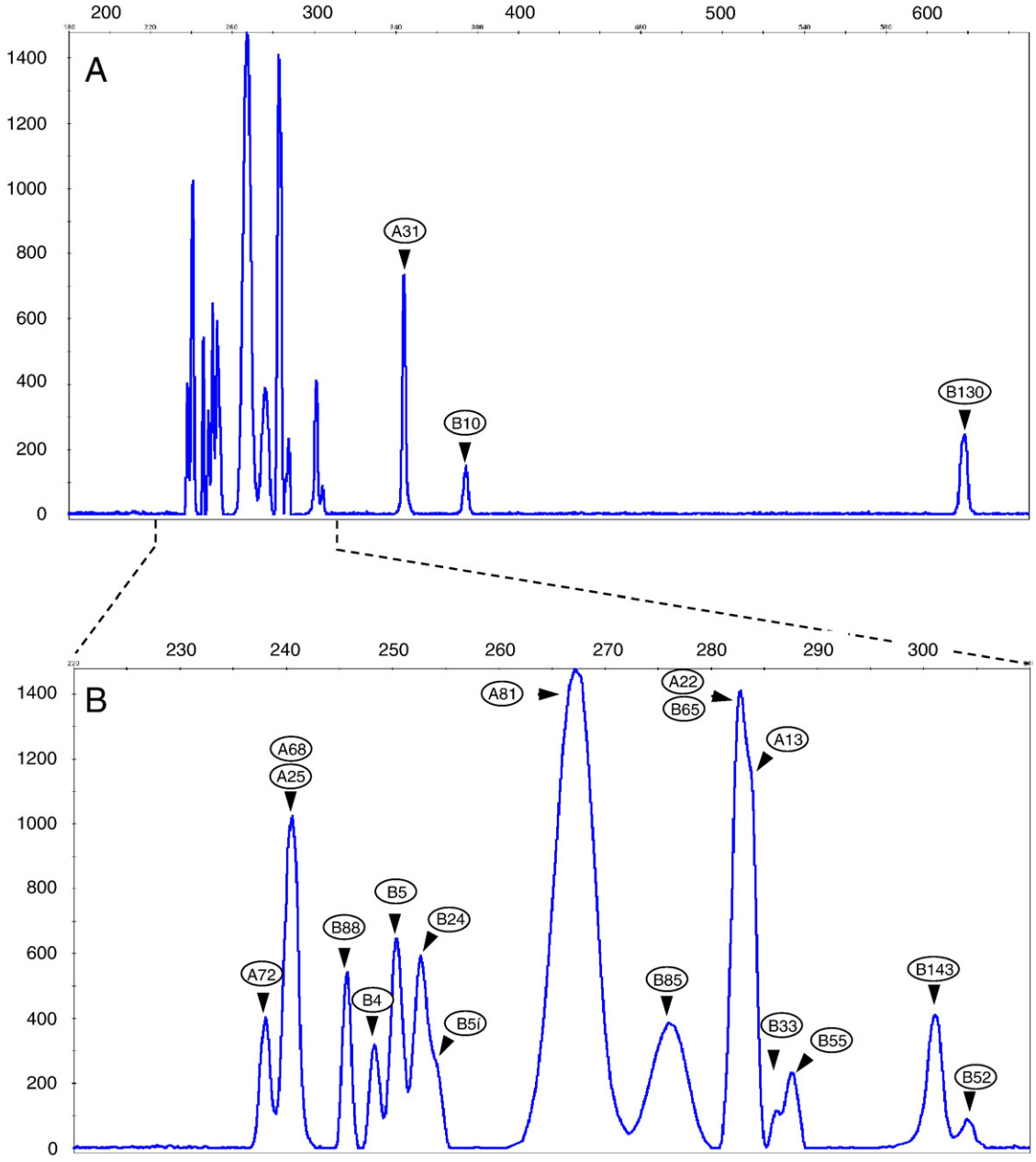

Fig. 2. Capillary electrophoresis fingerprint of ITS1-PCR fragments from 19 fungi strains (Table 1) in non-denaturing conditions (CE-SSCP). A: total fingerprint; B: up-sizing to 225–310 apparent bp. Axes are as in Fig. 1.

Electrophoresis was performed with POP4™ polymer (Applied Biosystems, Courtaboeuf, France), at 60 °C for 45 min. Injection time and voltage were 10 s and 3 kV respectively.

2.6. CE-SSCP

A 1 µl of 25-fold diluted PCR product was mixed with 10 µl formamide Hi-Di (Applied Biosystems, Courtaboeuf, France), 0.2 µl standard internal DNA molecular weight marker Genescan-400 HD ROX (Applied Biosystems, Courtaboeuf, France), 0.5 µl NaOH (0.3 M). The sample mixtures were denatured at 95 °C for 5 min and immediately cooled on ice before loading on the instrument. The non-denaturing polymer consisted of 5% CAP polymer, 10% glycerol and 3100 buffer (Applied Biosystems, Courtaboeuf, France). The injection time and voltage were set to 22 s and 6 kV, the migration time was set at 35 min at 32 °C. To

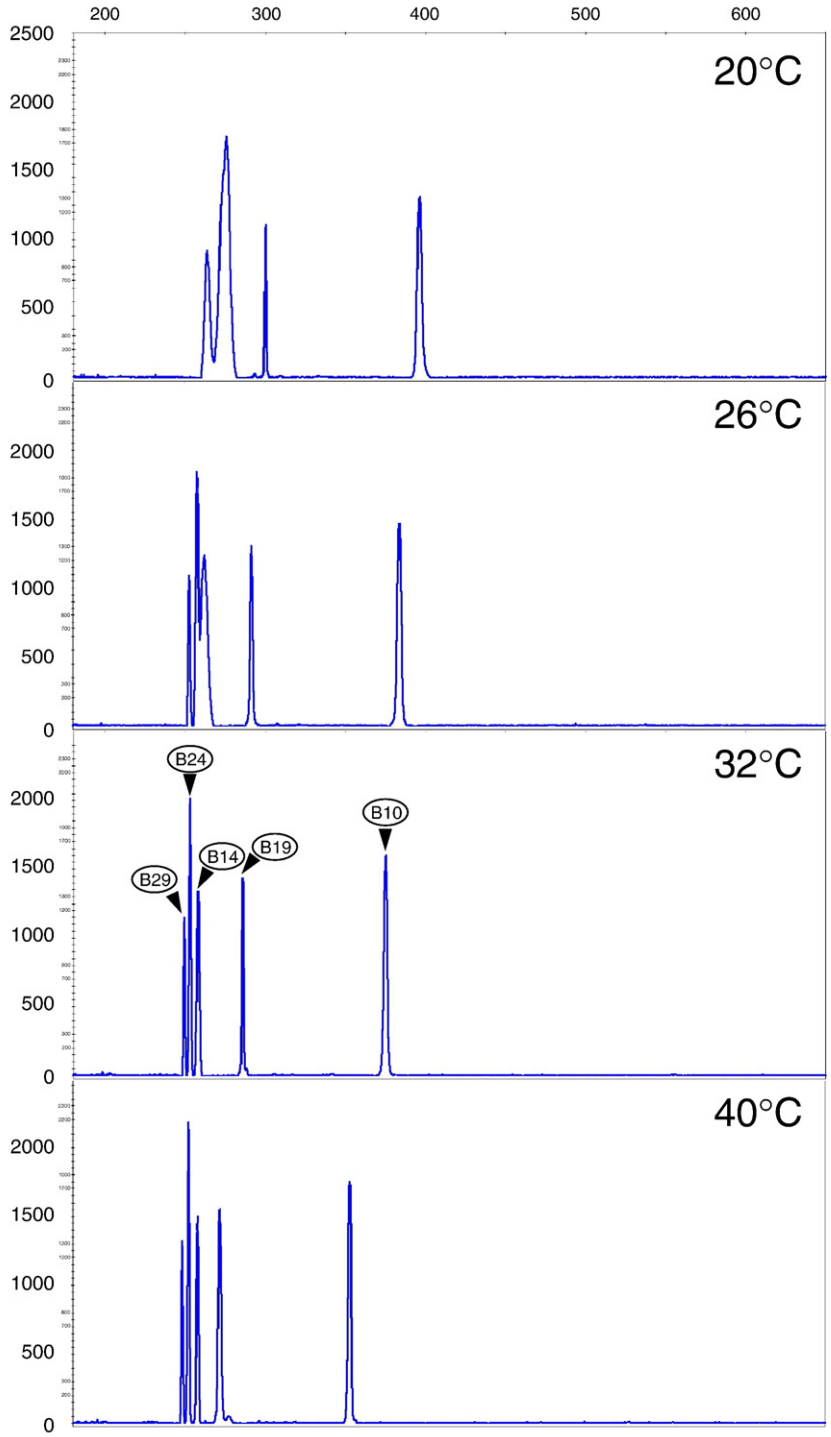

Fig. 3. CE-SSCP fingerprints of ITS1-PCR fragments from 5 fungal strains (see Table 1) at 20, 26, 32 and 40 °C. Axes are as in Fig. 1.

improve the information signal detection of CE-SSCP, migration temperatures were tested from 20 °C to 40 °C, by 2 °C steps.

2.7. Data analysis

CE-SSCP and CE-FLA profiles were analyzed with the GeneMapper™ Software Version 3.7 (Applied Biosystems, Courtaboeuf, France), using Genescan 400HD ROX or 500 ROX respectively as a size standard for peak pattern alignment.

3. Results

3.1. CE-FLA and CE-SSCP analysis of individual fungal strains

To study sensitivity and reproducibility of the two CE techniques, we tested the mobility values of isolated-fungi ITS1 region. For the CE-FLA, all the tested strains produced a single peak, which size varied between 230 and 567 b (Table 1). An excellent reproducibility was observed for most of the strains

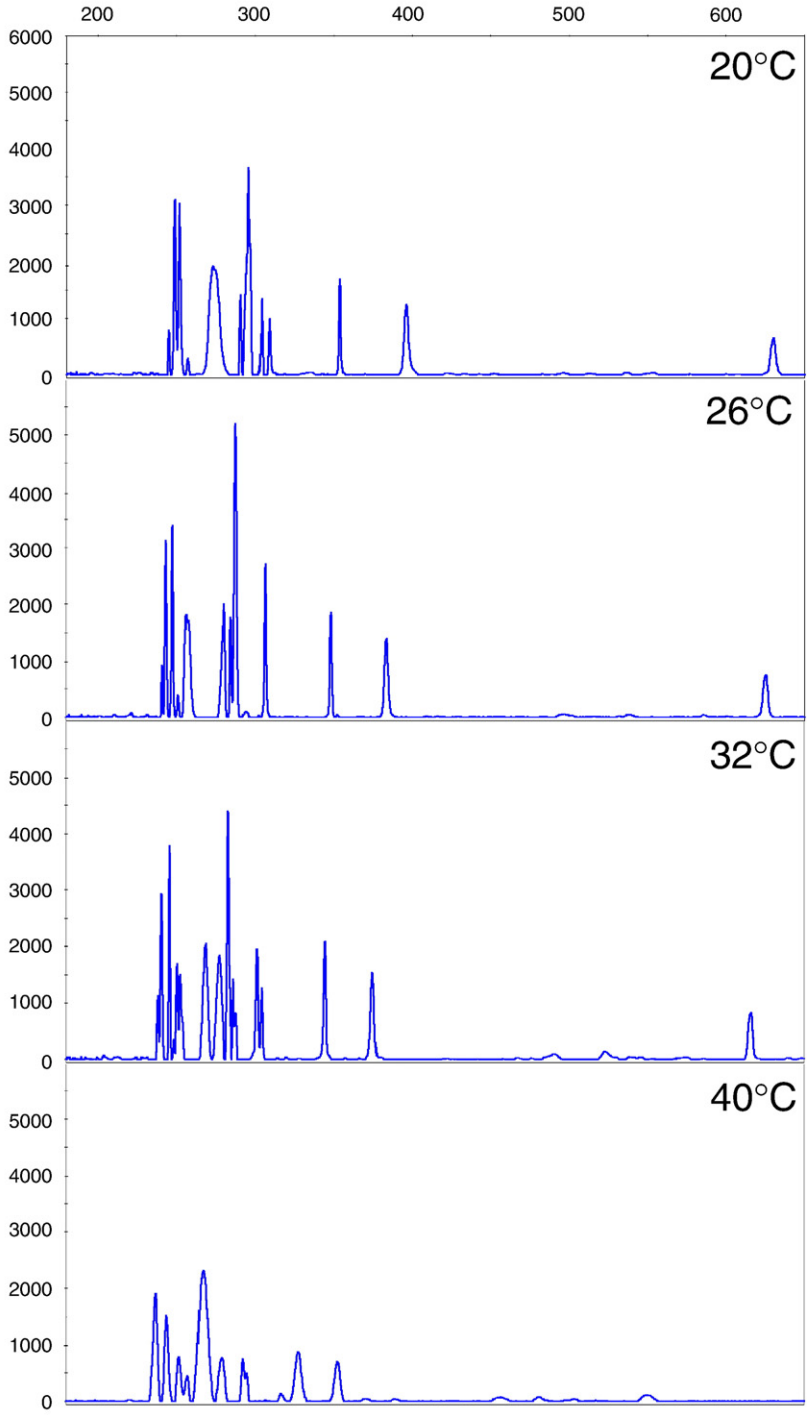

Fig. 4. CE-SSCP fingerprints of ITS1-PCR fragments from the 19 fungal strains mixture (Table 1) at four different temperatures. Axes are as in Fig. 1.

(Standard Deviation SD < 0.2 b). The ITS1 region of the strain B130 migrated out of the range of the internal standards used here, which was reflected in a high SD (0.937).

The CE-SSCP separates the amplicons according to their size and conformation (Orita et al., 1989). The migration size was assigned according to the standard DNA molecular weight marker

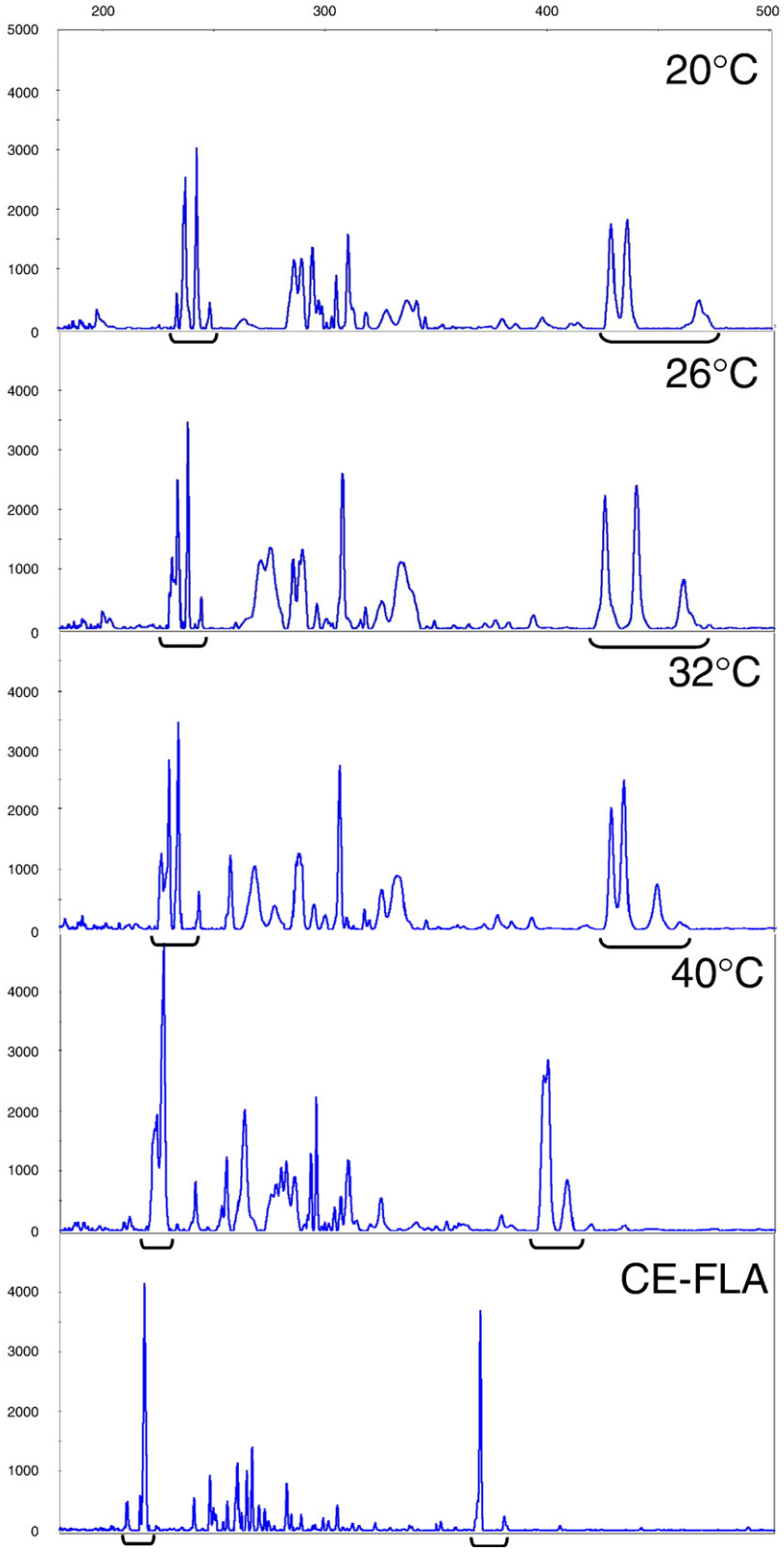

Fig. 5. CE-FLA and CE-SSCP patterns of alpine grassland soil at four different migration temperatures. Axes are as in Fig. 1.

(Table 1). Most of the strains were resolved for more than 1 b, and the SD was less than 1 b (except for the strain B130, out of size range). All the CE-SSCP profiles of the ITS1 fragments from isolated strains displayed a single peak, except the strain B5 which displayed a double one (250.54 and 254.24 b). However, the strain B5 displayed a single peak in the CE-FLA profile, indicating that there is no size polymorphism. The second peak observed in CE-SSCP may be due to two main reasons. Firstly, ITS1 of the B5 strain may have the same sequence but differently folded; this possibility was already observed (Atha et al., 2001) and previously proposed for prokaryotic 16 S rRNA genes (King et al., 2005; Sliwinski and Goodman, 2004). Secondly, the two peaks could represent sequence variations among the individuals of the isolate.

3.2. CE analysis of a PCR-DNA mix from 19 strains

In order to test the resolution of the two CE techniques, we imitated a fungal ecosystem by mixing the amplified DNA from the 19 alpine strains (Table 1). The peaks were assigned using the data from individual strains. As shown in Fig. 1, the CE-FLA profile displays 17 peaks of which 2 present shoulders (about 233 and 258 b). The assignment of the different strains was straight forward for the sharp peaks. The peaks at 233 and 258 b probably represented the pairs A25 (233.5 b)-A72 (232.5 b) and B33 (257.46 b)-B65 (258.30 b). Fragments which size differed in 1 b remained thus unresolved.

When CE-SSCP was conducted at 32 °C (see below), the profile displayed 15 peaks (Fig. 2), ranging from 238.17 to 617.57 b. The peaks assignment was also straight forward even for overlapping peaks. For example, the shoulder of the B24 peak (252.67 b) was probably due the second peak of the B5 strain. As for CE-FLA, 2 peaks presented a shoulder (at 240.39 and 282.91 b). These peaks corresponded to 2 (A25, A68) and 3 strains (A13, A22, A65) respectively, as shown in Fig. 2.

In this experiment, CE-FLA resolved 17 peaks *vs*. 15 for CE-SSCP. Moreover, the strains unresolved in CE-FLA were resolved in CE-SSCP and *vice versa*. For the mixture used here, FLA seemed to be superior for evaluating fungal diversity.

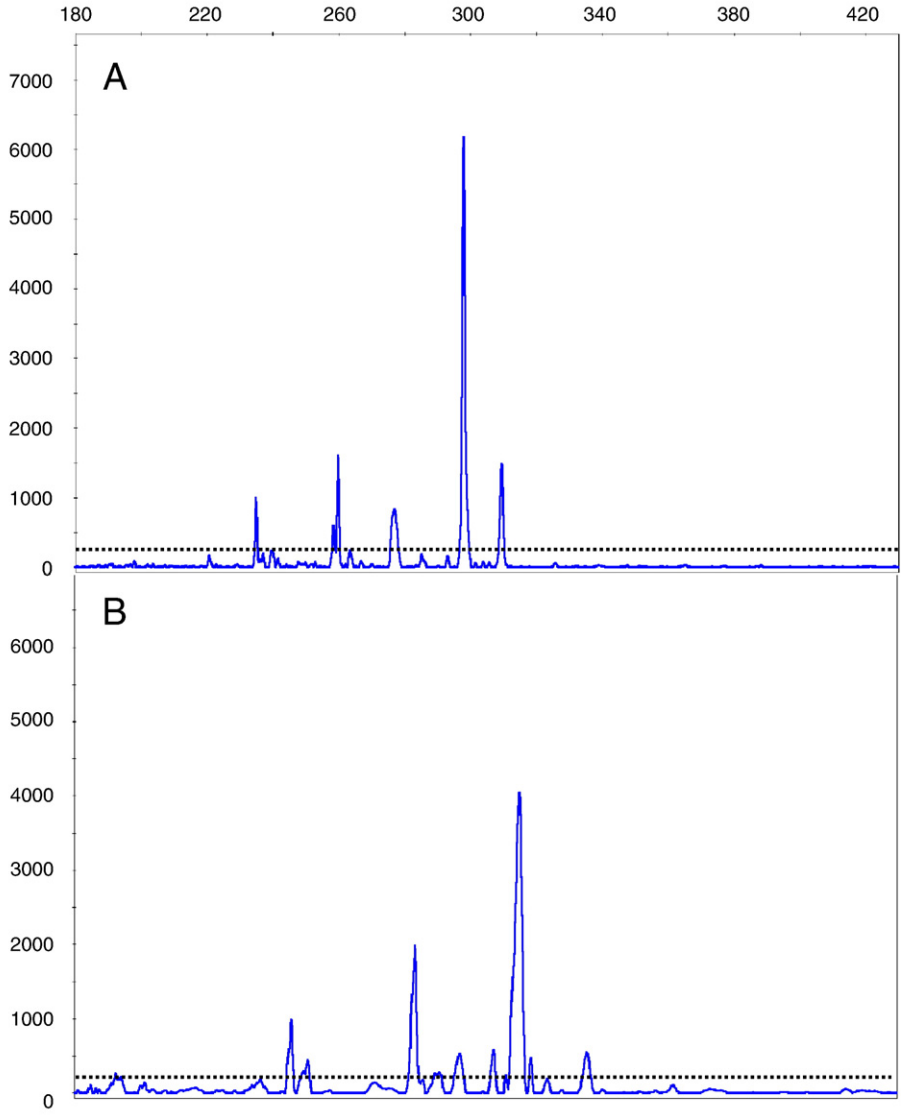

Fig. 6. CE-FLA (A) and CE-SSCP (B) patterns of infiltration basin sediments. Dot-line indicates 250 rfu. Axes are as in Fig. 1.

3.3. Temperature effects on SSCP

Migration temperature is known to have a strong impact on the mobility of a given DNA fragment. We thus tested a range of migration temperature to improve CE-SSCP. The most representative temperatures tested (*i.e.* 20, 26, 32 and 40 °C) are presented in Figs. 3, 4. Globally, the increase in temperature led to a drop of apparent size and better peak sharpness for isolated strains (data not shown), fungal-strain DNA mixes and soils. These changes resulted in an improvement of peak resolution at higher temperature (32 and 40 °C). We first analysed a 5-strain mixture (Table 1, Fig. 3), which included two strains (B29, B24) separated by 1 b (base), and a third (B14) separated by 6–7 b at 32 °C. These three strains formed only two peaks at 20 °C, but three peaks were resolved at 26 °C, with the best sharpness at 32 °C. Results obtained for the PCR-DNA mixture of 19 strains (Fig. 4) showed similar pattern for low temperatures (20 and 26 °C; 12 and 11 peaks respectively). At 32 °C, 15 sharper peaks were detected and only 9 were observable at 40 °C. For alpine soil, the number of peaks was smaller at lower temperatures, while the best resolution appeared to be achieved at 40 °C (Fig. 5), as observed for the mixture of 19 strains. However, the internal standard markers fused at the extreme temperatures: the 100 and 120 b markers formed only one peak at 20 °C, and the 280 and 290 b markers combined at 40 °C, making the analysis less accurate. Based on these results, a migration temperature of 32 °C for CE-SSCP is the best compromise.

3.4. CE analysis of environmental soil samples

To validate the use of these fingerprint methods, two environmental samples, *i.e.* alpine grassland soil and infiltration basin sediments, were analyzed and compared. The FLA pattern showed more and smaller peaks than the SSCP pattern for alpine soil as shown in Fig. 5. However, considering raw data, the SSCP pattern of the alpine soil displayed an increased baseline, which was absent in FLA (Supplementary Fig. 1), a phenomenon already reported for bacteria SSCP (Loisel et al., 2006).

The CE profiles from the infiltration basin sediments (Fig. 6) were quite different from those of the alpine soil for both studied methods. Firstly, infiltration basin sediments displayed fewer peaks, and secondly the peak sizes were quite different from those of the alpine soil. While we did not perform any further study on fungal diversity, these results support the expected difference in the fungal phylogenetic structure between these soils. When comparing the CE-FLA with the CE-SSCP profile setting 250 rfu as lower line, the later showed more peaks than the former. Moreover, the baseline was higher than in the alpine soil (Supplementary Fig. 2). Thus, in this ecosystem SSCP was superior to FLA as indicator of diversity.

4. Discussion

The assessment of soil microbial communities was profoundly improved by the detection of non-cultivated organisms using molecular markers, but the procedures relying on cloning are expensive and time consuming. In the framework of community dynamics or xenobiotic impact studies, where many samples have to be analyzed, the thoroughness of such methods is not required. Thus, in this case, the simplest fingerprinting methods, like FLA and SSCP, are preferable.

4.1. Choice of SSCP and FLA

Although T-RFLP is widely used and seems to be more efficient due to better phylotype discrimination, the use of restriction enzymes involves some limitations to high-throughput. Several enzymes should be used to ensure the digestion of a maximum of fragments (Avis et al., 2006), implying at least three independent digestions and independent CE-runs, with the concomitant increase in cost and time of analysis. Furthermore, the potential partial digestions may introduce biases (Egert and Friedrich, 2003; Osborn et al., 2000). However, the two methods described here necessitate only three steps: DNA extraction, PCR and electrophoresis. Moreover, CE-FLA and CE-SSCP separate shorter fragments to T-RFLP and ARISA, which reduces PCR and migration time (1 h 30 and 25 min respectively). Finally, selected samples with SSCP and FLA should also be approached by T-RFLP, cloning-sequencing approaches or by the 454 technology (Goldberg et al., 2006; Margulies et al., 2005).

4.2. Resolution of isolated strains

We tested the CE-FLA and CE-SSCP for the study of 22 fungal strains isolated from alpine soil, with ITS1-PCR fragments ranging from 230 to 567 b in FLA and 208 to 617 b in SSCP. Twenty one of these fragments were in the optimal range for SSCP. Most of the SD values were less than 0.2 b for FLA and 1 b for SSCP, indicating the excellent reproducibility of both methods except for the B130 strain, which was out of scale (>400 b) and displayed a higher SD. The high reproducibility of CE-SSCP and FLA make possible the construction of migration database for the phylotyping of environmental samples.

4.3. Resolution of mimicked ecosystems and soils

In the framework of microbial community analyses, all the fingerprinting methods display the same limitation: one peak may correspond to several phylotypes (Figs. 1 and 2). In environmental samples, a higher phylogenetic diversity with different abundance of phylotypes is expected. In this case, it is thus conceivable that there will be many co-migrating or closely migrating phylotypes. This hypothesis was supported by the patterns of alpine soil and polluted sediments. Firstly, the FLA pattern displayed many more minor peaks than the SSCP pattern (Figs. 5 and 6). Secondly, the raw data of SSCP showed an increasing of baseline (Supplementary Figs. 1 and 2). This baseline was previously observed for bacterial SSCP and was proposed to result from the fusion of closely migrating phylotypes and thus to carry information on bacterial diversity (Duthoit et al., 2003; Duthoit et al., 2005; Loisel et al., 2006).

However, further investigations should be done to understand its significance on fungal community analysis.

The SSCP and FLA showed good performances, resolving at least 15 of the 19 strains from the mixture (Figs. 1 and 2). SSCP seemed to be less efficient than FLA for the 19 strain mix and for alpine soil. Yet, in the case of infiltration basin sediments, our results (Fig. 6) support the theoretical superiority of SSCP, *i.e.* phylotypes sharing the same size in CE-FLA may be resolved because of the differences in sequence. Finally, in alpine soil, SSCP displayed more peaks than FLA in extremity regions of electrophoregram (Fig. 5, horizontal parenthesis), suggesting that phylotypes with these sizes were well resolved. This does not allow us to support SSCP instead of FLA, and it appears that they rather should be used in parallel.

Here, CE-FLA and CE-SSCP were able to discriminate two soils of different origin as shown in Figs. 5 and 6, supporting the assertion that their information provides a snapshot of microbial diversity in a given environment. Although these techniques do not directly supply an exhaustive inventory of species, they enable comparisons of many different samples. Moreover, they rapidly provide results and are cheaper than other fingerprinting and sequencing methods.

4.4. CE-FLA and CE-SSCP displayed a similar efficiency

We use CE-SSCP in our lab as routine analysis because of its shorter run time and its better suitability for multiplexing with bacteria. The bacterial molecular marker (the V3 region of the gene coding for the 16 S rRNA) displays almost no length polymorphism (two peaks in FLA), precluding FLA analysis. Thus, with differential labelling (FAM for fungi, HEX for eubacteria), the two communities may be analysed in a single CE-SSCP run with the concomitant reduction of costs. It may even be possible to follow two other markers, for instance, one labelled with NED for Archea and another labelled with VIC for functional genes.

4.5. Effect of temperature on SSCP

The SSCP is based upon the three-dimensional structure of DNA fragments. This structure is strongly linked to the sequence composition but also to temperature (Atha et al., 2001; Holmila and Husgafvel-Pursiainen, 2006; Kuhn et al., 2005). We thus tested this crucial parameter in order to optimize this method. The increase of temperature resulted in a drop of migration time. The study of a set of isolated strains showed that their apparent sizes reduced with rising temperatures and varied among strains (Figs. 3, 4). In most of the cases, the apparent size of the amplicon was higher than the size detected by FLA (Table 1) and decreased with the increase of temperature (Figs. 3–5) probably reflecting a collapse of the conformation. This phenomenon has already been highlighted using DNA-folding analysis (Atha et al., 2001). They showed that CE-SSCP mobility of different fragments converges with the rise of temperature but also that these mobility values were sequence dependent. In other words, fragment migrations reduce in different ways according to their sequence. In our experiments, the temperature chosen was 32 °C. This temperature seems to be the best compromise between resolution of complex samples and marker fusion.

4.6. CE-FLA and CE-SSCP for environmental analysis

Here, we compared two fingerprinting methods and assessed their advantages and limitations in the framework of fungal diversity studies. In such studies, the sampling strategy should be designed so as to take into account the high spatial heterogeneity of soil, as described previously (Schwarzenbach et al., 2007). Although fingerprinting analyses are frequently used in microbial diversity studies, there is no consensus for CE-SSCP pattern analysis. At present, many studies convert CE profiles into matrices of peak presence/absence. This approach has been used on molecular fingerprint studies of prokaryotes (Schwieger and Tebbe, 1998). Such matrices have also been used for the calculation of Euclidean distances or Jaccard coefficients, coupled or not with Neighbour-Joining clustering methods and dendrogram construction (Duthoit et al., 2005; Wenderoth et al., 2003). However, taking into account the peak intensity allows explaining these profiles in a more realistic way. The CE-signal expresses presence/absence as well as relative abundance of phylotype classes in the framework of microbial diversity studies. CE-patterns may be converted in frequency matrices allowing the calculation of diversity indices (Duthoit et al., 2003; Duthoit et al., 2005) and also submitted to PCA analysis (Ranjard et al., 2001; Sliwinski and Goodman, 2004). These approaches can also be used for the fungal CE-SSCP method described here.

In conclusion, FLA and SSCP do not reach the sensitivity of T-RFLP, but provide realistic diversity snapshot, high reproducibility and phylotyping by database creation. Moreover, they combine all requirements for high-throughput: simplicity, reproducibility, quickness, and multiplexing with limited costs. The development of high-throughput molecular signature methods should facilitate studies on several issues of microbial ecology.

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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.mimet.2007.10.005.

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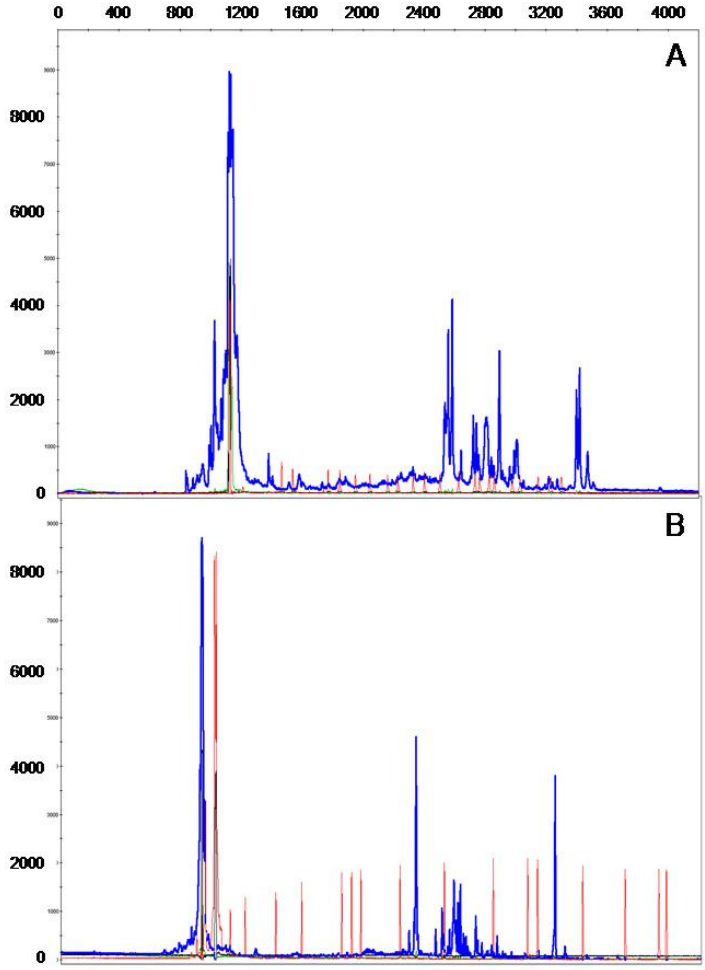
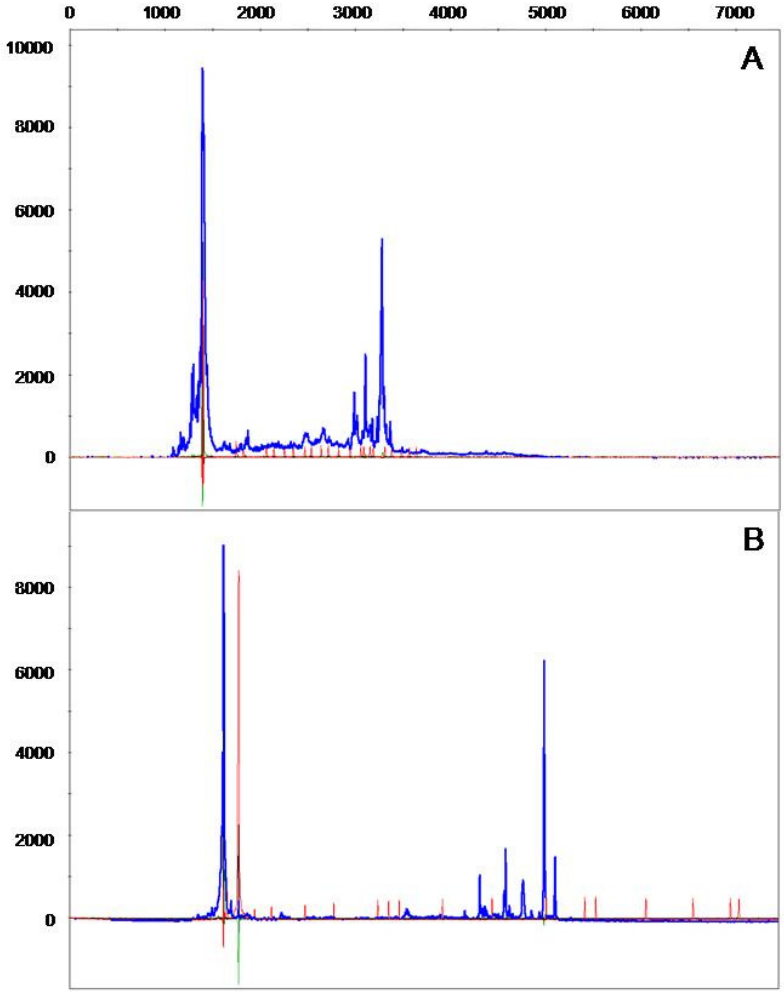
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. SCCP of alpine soil at 32°C (A) and FLA (B) profiles. Blue : ITS1, Red: Rox 400HD Size Markers. The y-axis is relative fluorescence units (rfu), the x-axis is the elution time represented in "datapoint".



Supplementary Figure 2. SCCP of infiltration basin at 32°C (A) and FLA (B) profiles. Blue : ITS1, Red: Rox 400HD Size Markers. The y-axis is relative fluorescence units (rfu), the x-axis is the elution time represented in "datapoint".

III. Principaux résultats et discussion

1. Optimisation de la CE-SSCP

Notre démarche s'est inscrite dans une volonté de mise en place d'une séquence expérimentale fiable et relativement simple pour une analyse routinière des communautés microbiennes. Dans un premier temps, nous avons constaté l'efficacité d'un kit commercial d'extraction d'ADN (Power Soil™ extraction kit, Laboratoires MO BIO) malgré la faible quantité de sol (0,250 g) utilisé pour l'extraction (cf. Article A). Nous avons également pu réduire la durée de l'amplification par PCR, tout en améliorant ses rendements (cf. Article A), répondant ainsi à notre désir d'améliorer la démarche expérimentale pour des analyses à haut-débit.

Ces études ont également montré l'incidence majeure de type d'ADN polymérase utilisé pendant l'amplification par PCR sur les profils moléculaires. En effet, alors que les ADN polymérases de type *Taq* peuvent ajouter une adénosine en 3' du brin synthétisé, l'activité exonucléase 3'-5' des ADN polymérase de type « proofreading » (activité de relecture) peut générer des pics multiples pour une seule souche microbienne en rognant un nombre variable de bases en 3' du brin synthétisé (Annexe A). Malgré l'augmentation significative des rendements et de la qualité de la PCR, cette particularité des « proofreading » peut conduire à une surestimation de la diversité microbienne (cf. Article A, Annexe A). En compromis, nous avons donc opté pour l'utilisation d'une ADN polymérase de type *Taq*, moins fidèle, mais dont les artefacts sont plus limités.

Enfin, la température de migration affecte profondément la conformation des ADN simples brins en conditions non dénaturante (Atha et al., 2001). Dépendant à la fois de la taille et de la séquence nucléotidique du fragment d'ADN, ces modification entraînent une variabilité de la mobilité électrophorétique brin-dépendante. En effet, nous avons observé la fusion de pics à 20 et 40°C. En conséquence, nous avons ici aussi établit un compromis de température de migration à 32°C de façon à limiter la fusion de certains pics tout en restant suffisamment résolutifs.

2. La CE-SSCP, une méthode robuste à haut-débit

Bien que les méthodes d'empreinte moléculaire permettent de caractériser la structure génétique des communautés, elles ne permettent pas d'identifier les espèces de ces communautés lorsque celles-ci sont complexes, ce qui est le cas dans le sol. Cette limitation provient d'une part, de la nécessité de disposer d'une banque de référence de mobilité électrophorétiques des souches microbiennes pour un marqueur moléculaire donné, et d'autre part, du fait qu'un pic dans le profil moléculaire ne représente pas forcément qu'une seule souche microbienne (Kirk et al., 2004; Loisel et al., 2006). A l'échelle de la communauté, les techniques d'empreintes moléculaires ne peuvent donc être appliquées que dans le cadre d'études comparatives visant à établir des co-variations entre la structure des communautés microbiennes et les conditions environnementales.

Dans ce contexte, il n'est pas nécessaire de disposer d'une méthode exhaustive, mais plutôt d'une méthode **suffisamment fiable et discriminante**, **générant des données comparables,** pour détecter des variations dans la structure des communautés. Les études du présent chapitre démontrent bien que la CE-SSCP répond à ces critères, de par sa haute reproductibilité, quelque soit le manipulateur, le type d'extraction d'ADN et les caractéristiques de la PCR, et par une discrimination suffisante des profils moléculaires obtenus de sols différents (Articles A et B). Bien que la CE-FLA (équivalent de l'ARISA), testée sur les communautés fongique par le biais de l'ITS1, ait montré une performance similaire (Article B), il nous était difficile appliquer cette méthode aux communautés bactériennes, ne disposant pas d'un marqueur moléculaire bactérien répondant aux exigences de la FLA et de l'électrophorèse en capillaire à savoir un marqueur court et hautement polymorphique en taille.

3. Des perspectives pour la CE-SSCP

L'application de cette méthode aux communautés bactériennes et fongique s'est révélée extrêmement aisée à mettre en œuvre. Nous avons donc rapidement pu appliquer cette méthode aux communautés de crenarchaeotes. Il serait donc envisageable de suivre d'autres types de communautés ou encore des gènes fonctionnels. En outre, l'utilisation de l'électrophorèse en capillaire permet de suivre plusieurs fluorophores dans un même capillaire. En marquant les phylotypes fongiques, bactériens et crenarchaeotes avec des fluorophores différents, nous avons pu ainsi réduire par 3 le nombre et le temps de migrations.

L'analyse des profils moléculaires générés par la CE-SSCP s'est révélée problématique en utilisant le logiciel fournit par le constructeur du séquenceur. Développé pour des méthodes d'empreintes moléculaires générant des donnés discrètes (microsatellites, AFLP [Amplified Fragment Length Polymorphism], etc…) ce logiciel forme artificiellement des classes à partir des données continues générés par la CE-SSCP, entrainant ainsi une perte d'information considérable (Loisel et al., 2006). Nous avons donc dû développer nos propres outils d'analyse. Dans un premier temps, nous avons fait appel à Benjamin Bonnes (ingénieur informaticien à Cap Gemini) afin de développer une application permettant d'extraire les données brutes du séquenceur sous format numérique. Dans un deuxième temps, nous avons fait appel aux compétences en programmation de Philippe Choler (LECA), dans le but de lisser et normaliser ces données brutes, nous permettant ainsi de prendre compte l'ensemble du profil pour comparer les communautés.

Dans le cadre d'études comparatives, les techniques d'empreintes moléculaires se doivent d'être hautement reproductible, suffisamment discriminantes, et à haut débit. Nous avons réussi à répondre à l'ensemble de ces critères en utilisant la CE-SSCP, en optimisant chaque étape expérimentale, et en développant des outils informatiques adaptés aux données générées par cette méthode. Bien que notre démarche engendre des données approximatives, elle permet néanmoins de les confronter de façon fiable, la rendant tout à fait appropriée pour des études comparatives.

Chapitre II – Effet des régimes d'enneigement sur la dynamique des communautés microbiennes des sols alpins

I. Problématique et démarche scientifique

1. Contexte général

Dans le contexte actuel du réchauffement climatique, il est nécessaire de prédire l'impact de l'augmentation de la température sur les flux de carbone émanant du sol. Les sols constituent des stocks de carbone, dont l'importance est fortement dépendante des conditions environnementales. Les plus importants stocks de carbone organique sont principalement localisés dans les régions de haute latitude/altitude, où les faibles températures inhibent l'activité des micro-organismes de façon indirecte, par recrutement d'une végétation à litière récalcitrante, et directe, par inhibition des activités enzymatiques (revu par Davidson et Janssens, 2006). Une augmentation des températures pourrait réactiver l'activité microbienne, et en conséquence augmenter les flux de carbone sortants, faisant passer ces sols d'un statut « puits » à un statut « source » de carbone (Oechel et al., 2000).

Dans les écosystèmes alpins, l'effet du réchauffement global sur les stocks de carbone du sol demeure difficile à prédire. En effet, **la disparité des régimes d'enneigement** influence les communautés végétales aussi bien au niveau spécifique que fonctionnel (Körner, 1995; Choler, 2005). Ainsi, des régimes d'enneigement faibles favorisent la présence d'espèces stress-tolérantes dont la litière est plus récalcitrante que les espèces à croissance rapide présentes dans les systèmes nivaux. Cette différence est susceptible d'avoir des conséquences significatives sur le cycle du carbone (cf Introduction §V.1, Baptist, 2008). De plus, le manteau neigeux protège les sols des gels hivernaux, maintenant ainsi l'activité métabolique des micro-organismes. Le taux de recyclage du carbone apparait donc plus élevé dans les systèmes nivaux (Brooks et al., 1997; Fisk et al., 1998), mais aussi inconstant au cours de l'année: la durée des gels hivernaux, la disponibilité des ressources, ainsi que l'état d'avancement de la saison de végétation peuvent profondément influer sur la respiration hétérotrophe du sol (Schinner, 1983; Brooks et al., 1997; Monson et al., 2006; Baptist, 2008).

En raison de l'hétérogénéité des régimes d'enneigement, les sols alpins sont soumis à des **rythmes saisonniers plus ou moins contrastés**, susceptibles d'agir de façon plus ou moins marquée sur la succession des communautés microbiennes. Cette dynamique de la microflore serait potentiellement responsable des variations annuelles des flux de CO_2 émanant des sols précédemment mis en évidence (Schinner, 1983; Brooks et al., 1997; Lipson et al., 1999). Bien que la dynamique des communautés microbiennes alpine ait déjà été rapportée dans des pelouses thermiques (dominées par *Kobresia myosuroides* et *Dryas octopetala*; Schadt et al., 2003; Lipson and Schmidt, 2004), l'effet des régimes d'enneigement sur cette dynamique reste mal connue. Les communautés microbiennes étant impliquées dans le recyclage de la matière organique (cf. Introduction §I.3), l'étude de l'impact du régime d'enneigement sur ces communautés permettrait une meilleure compréhension des processus biogéochimiques dans ces milieux. Cependant, de telles études sont rares (mais voir Nemergut et al., 2005; Bjork et al., 2008), et bien qu'elles aient pu mettre en évidence un certain effet du régime d'enneigement sur la structure des communautés microbiennes, leur composition en espèce demeure pauvrement caractérisées.

Les écosystèmes alpins sont contrastés à une échelle spatiale (*e.g.* opposition crête et combe) et temporelle (*i.e.* cycles de gel/dégel ou d'humidité/sécheresse, forte amplitude thermique annuelle) et constituent donc un modèle d'étude approprié pour identifier les facteurs prépondérants impliqués dans l'assemblage de communautés microbiennes. La caractérisation de la dynamique des communautés microbiennes alpines le long d'un gradient d'enneigement constitue donc un premier pas vers une approche plus globale des cycles biogéochimiques dans les écosystèmes froids. Elle peut en outre faire émerger des règles fondamentales sur l'assemblage de ces communautés valables dans l'ensemble des écosystèmes terrestres.

2. Objectifs de l'étude

Notre étude s'est concentrée sur des habitats se situant aux deux extrêmes du gradient d'enneigement : des situations thermiques présentant un enneigement hivernal faible voire inexistant (crêtes, fonte des neiges en Mai), et des situations nivales présentant un manteau neigeux plus persistant (combes, fonte des neiges en Juin ; Fig. 13). Cette différence d'enneigement influence profondément la durée de la saison de végétation et, en conséquence, l'assemblage des communautés végétales dans ces deux situations (Fig. 13). Du fait de différences marquées de la nature du carbone entrant (récalcitrance de la litière) ainsi que dans les processus de décomposition/humification, ces deux habitats ont donc potentiellement

un comportement différent en matière de flux de carbone ; les situations thermiques sont pressenties comme étant des « puits » et les situations nivales comme des « sources » de carbone (cf Introduction §V.1, Baptist, 2008).

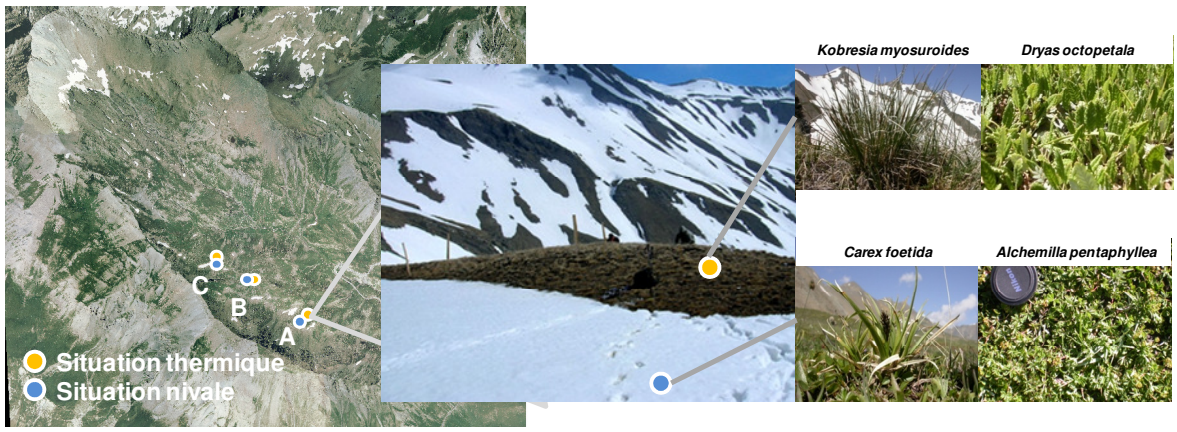


Figure 13 : Localisation des sites d'études et illustration des espèces végétales dominantes des situations thermiques (*K. myosuroides* et *Dryas octopetala*) et nivales (*Carex foetida* et *Alchemilla pentaphyllea*). *Image satellite : Meteo France, Photographies : C. Gallet et P. Choler.*

Notre objectif a donc été de caractériser la dynamique saisonnière des communautés microbiennes des sols de situations thermiques et nivales. Le présent chapitre s'articule donc autour de :

1. Un suivi sur deux années de la dynamique saisonnière des communautés microbiennes des situations thermiques et nivales en utilisant la CE-SSCP. Ce suivi a été également effectué par clonage/séquençage sur un site la première année ce qui nous a permis d'identifier les grands groupes bactériens (gène de l'ARN 16S), et fongiques (gène de l'ARN 28S) dominant ces habitats. Ces données de séquences nous ont aussi permis d'évaluer la diversité de ces communautés (Article C).
2. Une caractérisation plus fine de la composition des communautés bactériennes en utilisant nos données précédemment obtenues par clonage/séquençage. Nous avons utilisé des approches phylogénétiques de type NTI (Nearest Taxa Index) permettant de déterminer si les communautés bactériennes étaient phylogénétiquement groupées, *i.e.* composées d'un groupe d'espèces phylogénétiquement proches, ou dispersée, *i.e.* composées d'espèces phylogénétiquement diverses. Cet outil permet d'évaluer si les conditions environnementales agissent comme des filtres sur l'assemblage des communautés bactériennes (Annexe B).
3. Une identification plus précise des espèces fongiques *via* nos banques de clones précédemment obtenues avec en complément le clonage/séquençage de la région ITS1. Cette

région a été utilisée en raison d'un référencement plus fourni dans les bases de données internationales. Les difficultés rencontrées pour l'analyse de ces jeux de données, notamment la mauvaise qualité des alignements multiples, étape indispensable à la construction d'une phylogénie, nous ont conduites à proposer une démarche alternative basée sur des alignements de séquences deux-à-deux et sur la théorie des graphes (Article D).

4. Un suivi de la dégradation de la matière organique récalcitrante (polyphénols), et son influence sur la structure des communautés microbiennes (suivi par CE-SSCP), et sur les cycles biogéochimiques (dénitrification, flux de CO_2), dans des sols de situation thermique soumis à de basses températures (0 et -6°C) (Annexe C).

II. Contribution scientifique

Article C: Zinger L., Shahnavaz B., Baptist F., Geremia R.A., Choler P. (2009): Microbial diversity in alpine tundra soils correlates with snow cover dynamics. *ISME J.*, 10p. doi:10.1038/ismej.2009.20.

Article D: Zinger L., Coissac E., Choler P., Geremia R.A.: Microbial communities assessed by graph partitioning approach: a study of soil fungi in two alpine ecosystems. *submitted in Appl. Environ. Microbiol.*

Annexe B: Shahnavaz B., Zinger L., Lavergne S., Choler P., Geremia R.A.: Snow cover dynamics and phylogenetic structure of bacterial communities in alpine tundra soils. s*ubmitted in Environ. Microbiol.*

Annexe C: Baptist F., Zinger L., Clement J.C., Gallet C., Guillemin R., Martins J.M.F. Sage L., Shahnavaz B., Choler P., Geremia R.A. (2008) Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach. *Environ. Microbiol.* **10**: 799-809.

Chapitre II – Article C

Microbial diversity in alpine tundra soils correlates with snow cover dynamics.

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ORIGINAL ARTICLE

Microbial diversity in alpine tundra soils correlates with snow cover dynamics

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The temporal and spatial snow cover dynamics is the primary factor controlling the plant communities' composition and biogeochemical cycles in arctic and alpine tundra. However, the relationships between the distribution of snow and the diversity of soil microbial communities remain largely unexplored. Over a period of 2 years, we monitored soil microbial communities at three sites, including contiguous alpine meadows of late and early snowmelt locations (LSM and ESM, respectively). Bacterial and fungal communities were characterized by using molecular fingerprinting and cloning/sequencing of microbial ribosomal DNA extracted from the soil. Herein, we show that the spatial and temporal distribution of snow strongly correlates with microbial community composition. High seasonal contrast in ESM is associated with marked seasonal shifts for bacterial communities; whereas less contrasted seasons because of long-lasting snowpack in LSM is associated with increased fungal diversity. Finally, our results indicate that, similar to plant communities, microbial communities exhibit important shifts in composition at two extremes of the snow cover gradient. However, winter conditions lead to the convergence of microbial communities independently of snow cover presence. This study provides new insights into the distribution of microbial communities in alpine tundra in relation to snow cover dynamics, and may be helpful in predicting the future of microbial communities and biogeochemical cycles in arctic and alpine tundra in the context of a warmer climate.

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Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: seasonal variations; SSCP; carbon cycle; global change

Introduction

Seasonally snow-covered soils account for 20% of the global land surface (Beniston *et al.*, 1996). It is largely assumed that these soils sequester large amounts of organic carbon (Davidson and Janssens, 2006), and that the mineralization of this carbon stock is of increasing concern in a warmer climate (Hobbie *et al.*, 2000; Oechel *et al.*, 2000; Melillo *et al.*, 2002). In arctic and alpine tundra, the duration of snow cover has dramatic impacts on ecosystem structure and functioning (Fisk *et al.*, 1998; Walker, 2000; Welker *et al.*, 2000; Edwards *et al.*, 2007). The high topographic complexity found in alpine tundra triggers strong landscape-scale snow-cover gradients, which in the short term strongly affects local climatic conditions. In the long term, it leads to striking differences in plant cover and ecosystem processes (Billings, 1973; Bowman *et al.*, 1993; Körner, 1999; Choler, 2005). Thus, alpine tundra offers ecologically relevant opportunities to assess the impact of snow on local climatic conditions and ecosystem processes (O'lear and Seastedt, 1994; Litaor *et al.*, 2001; Choler, 2005). Several studies have suggested that many key drivers of soil organic matter mineralization, such as soil temperature, soil moisture, and litter quantity and quality, vary in a conserved manner along snow cover gradients in alpine landscapes (Fisk *et al.*, 1998; Hobbie *et al.*, 2000). Concomitantly, other studies highlighted the seasonal shift of microbial communities and activities in dry alpine tundra (Lipson *et al.*, 1999; Schadt *et al.*, 2003; Lipson and Schmidt, 2004; Schmidt *et al.*, 2007). Given that increasing temperatures will influence the snow cover dynamics in the alpine tundra (Marshall *et al.*, 2008), mineralization processes and associated microbial communities will most likely be affected by these changes as well. However, alpine microbial communities are not well known, and only a few comparative studies of microbial community

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dynamics in relation to snow cover patterns have been reported (Zak and Kling, 2006; Björk *et al.*, 2008).

In this study, our main objective was to test for spatial (that is, plant cover and soil characteristics) and temporal co-variations between soil bacterial and fungal communities, and snow cover dynamics in alpine tundra. We compared two contrasted conditions in alpine tundra, namely early snowmelt (ESM) and late snowmelt (LSM) locations, for 2 years. The phylogenetic structure of bacterial and fungal communities was first assessed using single-strand conformation polymorphism (SSCP) (Stach *et al.*, 2001; Zinger *et al.*, 2007, 2008) and were further characterized by cloning/sequencing. The molecular diversity in microbial communities was examined at four different sampling periods: (i) May, in the presence of late winter snowpack in LSM or immediately after thawing in ESM; (ii) June, corresponding to snowmelt in LSM locations and the greening phase for ESM; (iii) August, when there is a peak of standing biomass; and (iv) October, during litterfall and just before the early snowfalls (Figure 1a).

Materials and methods

Sample collection and soil characterization

The study site was located in the Grand Galibier massif (French southwestern Alps, 45°0.05′N, 06° 0.38′E) on an east-facing slope. Microbial communities were studied in three sites (ESM A: 45° 1′48.47″N 6°13′50.14″W, B: 45°1′52.78″N 6°13′ 26.88″W, C: 45°1′54.35″N 6°13′19.32″W; LSM A: 45°1′48.34″N 6°13′50.20″W, B: 45°1′52.79″N 6°13′ 22.85″W, C: 45°1′53.67″N 6°13′26.73″W) each comprising neighboring LSM and ESM locations. For each site, the locations stand a few meters away (5–10 m) and the sites are separated by 200–500 m. The surface of each location is comprised between 50 and 100 m^2. Plant coverage and soil parameters are same among sites for a given location (ESM or LSM). Five spatial replicates for each plot at each date were collected from the top 10 cm of soil and sieved (2 mm). During the first year of the survey (2005–2006), only site B was sampled on 24 June, 10 August and 10 October 2005, and 3 May 2006. Sites A, B and C were monitored during the second year (2006–2007), and were sampled on 30 July and 2 October 2006, and 18 May 2007 (Figure 1a). Late-winter snow cover consisted of 1–2.5 m depth in LSM locations. Soil organic matter content was determined by loss on ignition (Schulte and Hopkins, 1996) in soil sampled in September. Soil texture was determined using standard methods by the Institute National de la Recherche Agronomique (Laboratoire d'Analyses des Sols, Arras, France). For each spatial replicate ($n=5$), 5 g of soil were mixed in 15 ml of distilled water to determine the pH. Differences of pH ($P<0.05$) between each point were determined by Tukey's test with the R software (The R Development Core Team, 2007).

CE-SSCP analysis of microbial diversity

Three replicates of soil DNA extraction were carried out for each sample with the Power Soil Extraction

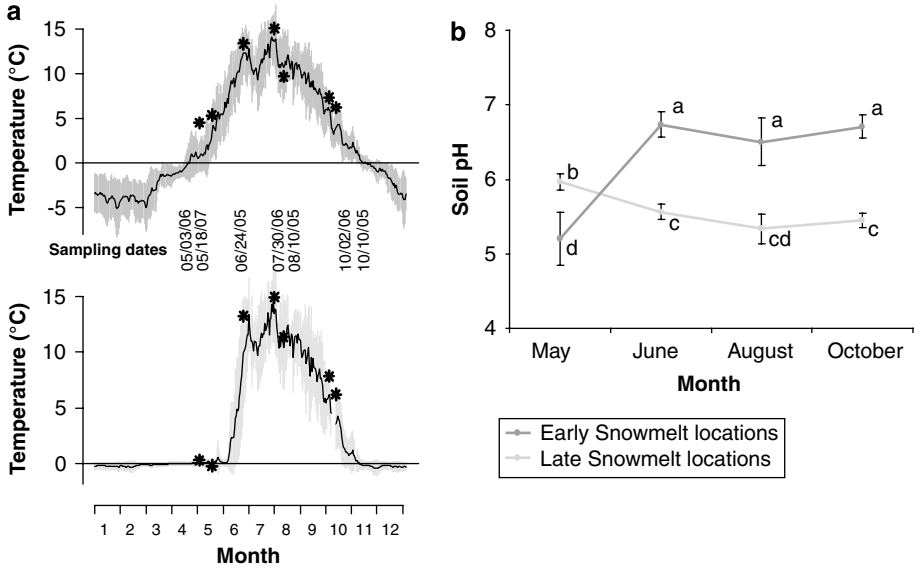

Figure 1 Temperature and pH in ESM (dark gray) and LSM (light gray). (**a**) Yearly course of daily mean (± s.e.) soil temperature at 5 cm belowground. Data are averaged over the period 1999–2007 and were recorded in two or three different sites depending on the year. Snowmelts occurred around 1 May in ESM and 40 days later (mid-June) in LSM. Daily mean soil temperatures corresponding to sampling dates, shown by stars, point out the typical climate conditions that occurred during the survey in both ESM and LSM locations. (**b**) Mean soil pH measured from June 2005 to May 2006 in the site B ($n=5$). Error bars indicate ± s.d. Both studied locations are significantly different throughout the year, and each of them revealed a significant shift of pH in May (indicated by lower-case letters, $P<0.05$).

Kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) according to the manufacturer's instructions. To limit the effects of soil spatial heterogeneity, 15 DNA extracts obtained from the five spatial replicates per location and date were pooled, rendering one DNA pool per location per date. This sampling and pooling strategy is in accordance with recent reports (Schwarzenbach *et al.*, 2007; Yergeau *et al.*, 2007a, b), and have been validated for fungal communities (June 2005 to May 2006, Zinger, unpublished data). The V3 region of 16S rRNA gene was used as the bacterial-specific marker using the primers W49 (5′-ACGGTCCAGA CTCCTACGGG-3′) and W104-FAM (5′-TTACCG CGGCTGCTGGCAC-3′) (Delbes *et al.*, 1998), whereas the ITS1 region, amplified with the primers ITS5 (5′-GGAAGTAAAAGTCGTAACAACG-3′) and ITS2-FAM (5′-GCTGCGTTCTTCATCGATGC-3′) (White *et al.*, 1990), was used as a fungal marker. PCRs (25 μl) were set up as follows: 2.5 mM of $MgCl_2$, 1 U of AmpliTaq Gold polymerase (Applied Biosystems, Courtaboeuf, France), 1 × of buffer provided by the manufacturer, 20 g l^{-1} of bovine serum albumin, 0.1 mM of each dNTP, 0.2 μM of each primer and 10 ng of DNA template. A 9700 dual 96-well sample block (Applied Biosystems) was used for thermocycling, with an initial denaturation at 95 °C for 10 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 15 s and extension at 72 °C for 15 s, and a final elongation at 72 °C for 7 min. The amplicons of each sample were then submitted to CE-SSCP as described earlier (Zinger *et al.*, 2007, 2008). The profiles obtained from CE-SSCP were normalized and compared by constructing dendrograms from Edwards' distance and Neighbor-Joining, with 1000 bootstrap replications. These analyses were carried out with the R software (R Development Core Team, 2007).

Clone library construction and analysis

Clone libraries were constructed for the samples from the site B (2005–2006). Bacteria communities were monitored using the 16S rRNA genes, amplified with 63F (5′-CAGGCCTAACACATGC AAGTC-3′) (Marchesi *et al.*, 1998) and Com2-ph (5′-CCGTCAATTCCTTTGAGTTT-3′) (Schmalenberger *et al.*, 2001). The 28S rRNA genes were amplified for fungal communities with U1 (5′-GTGA AATTGTTGAAAGGGAA-3′) (Sandhu *et al.*, 1995) and with nLSU1221R (5′-CTAGATGAACYAA-CACCTT-3′) (Schadt *et al.*, 2003). PCRs were carried out with 2.5 mM $MgCl_2$, 0.1 mM each ddNTP, 0.4 μM (bacteria) or 0.2 μM (fungi) each primer, 1 U AmpliTaq Gold polymerase, 1 × of buffer provided by the manufacturer, 20 g l^{-1} of bovine serum albumin and 10 ng of DNA of each location pool as a template. PCR was carried out as follows: initial denaturation at 95 °C for 10 min, 25 (bacteria) or 30 (fungi) cycles at 95 °C for 30 s, 54 °C (bacteria) or 53 °C (fungi) for 30 s and 72 °C for 1 min and 30 s, and final elongation at 72 °C for 15 min (bacteria) or 7 min (fungi). Eight independent PCR amplifications were carried out on each sample, pooled and cloned using a TOPO TA PCR 4.1 cloning kit (Invitrogen SARL, Molecular Probes, Cergy Pontoise, France). The titers of ligation were between 25 and 446 c.f.u. ng^{-1} of soil DNA. The transformation and sequencing were carried out at the Centre National de Séquençage (Genoscope, Evry, France). Approximately 350–380 sequences per library were obtained. Clones were identified using Ribosomal Database Project's Classifier (Cole *et al.*, 2003) for bacteria and BLAST (Altschul *et al.*, 1997) for fungi. Bellerophon (Huber *et al.*, 2004) was used to identify chimerical sequences. A multiple alignment for each kingdom was carried out with ClustalW (Chenna *et al.*, 2003) and cleaned by removing nucleotide positions with more than 30% of gaps and sequences smaller than 400 bp. After this cleaning step, 2226 sequences with 499 nucleotide positions for bacteria (GenBank accession nos. FJ568339–FJ570564) and 2559 sequences with 617 nucleotide positions for fungi (GenBank accession nos. FJ568339–FJ570564) were included in the phylotype composition and diversity analysis. We used pairwise distances and complete linkage method to cluster 700 randomly sampled DNA sequences of bacteria or of fungi. Sequences were then pooled according to different similarity thresholds (from 70 to 100%). For each sequence similarity level, we calculated the converse of the Simpson index to estimate the evenness of the profile of operational taxonomic unit (OTU) abundances (Smith and Wilson, 1996). The procedure was repeated 1000 times. All computations were carried out using the R software (The R Development Core Team, 2007).

Results

Characterization of ESM and LSM locations

The temperature of soil from ESM and LSM locations was determined for 7 years. ESM locations are characterized by shallow or inconsistent winter snow cover, leading to long periods of soil freezing (Figure 1a). In contrast, LSM locations exhibit long-lasting, deep and insulating snowpack almost 8 months per year, which leads to a fairly constant winter soil temperature around 0 °C (Figure 1a). In almost all the cases, the soil temperature during sampling was comprised between the usual temperatures for the season (Figure 1a). The contrasting snow cover environments are associated with marked variations in plant communities (Table 1) (Choler, 2005). LSM are dominated by low-stature species, such as *Carex foetida* (Cyperaceae) and *Salix herbacea* (Salicaceae), which must cope with a shorter growing season. Plant cover in ESM locations is more discontinuous and dominated by *Kobresia myosuroides* (Cyperaceae), a stress-tolerant turf graminoid, and *Dryas octopetala* (Rosaceae), a dwarf shrub. The upper soil layer in ESM locations

Table 1 Characteristics of LSM and ESM locations

	LSM situation	*ESM situation*
Plant cover		
Dominant species	*Carex foetida All.* *Alchemilla pentaphyllea L.* *Salix herbacea L.* *Alopecurus alpinus Vill.*	*Kobresia myosuroides* *Dryas octopetala* *Carex curvula All. subsp. rosae* *Gilomen*
Soil characteristics		
Soil classification	Stagnogley enriched in clay	Alpine Ranker
% Organic matter (top soil 10 cm)	8.7 ± 2.5	15.7 ± 4.7
Grain size analysis		
Clay (<2 μm)	34.6 ± 2.6	9.7 ± 0.5
Silt (2–50 μm)	59.0 ± 3.5	41.4 ± 1.0
Sand (50–2000 μm)	6.5 ± 1.7	48.6 ± 1.2

Abbreviations: ESM, early snowmelt; LSM, late snowmelt.
Values presented here are mean ± s.d.

has a higher soil organic matter content than that in LSM locations, but the carbon stock is lower due to shallower soils (Table 1). Soil pH is stable and higher in ESM throughout the year, except in winter when ESM soils become more acidic than LSM soils (Figure 1b).

Effects of snow cover patterns on temporal microbial community structure revealed by molecular profiling

The microbial communities were monitored from August 2006 to May 2007 at three sites, each including ESM and LSM locations. The structure of the microbial communities was assessed using capillary electrophoresis-based SSCP (CE-SSCP) by amplifying the V3 region of *ssu* gene using PCR for bacteria and the ITS1 (internal-transcribed spacer 1) for fungi. Distance trees based on the SSCP profiles revealed a significant difference within bacterial and fungal communities between ESM and LSM. This pronounced difference was noticed for all study sites and sampling dates (Figure 2a). The similar pattern for the three sampling sites indicates

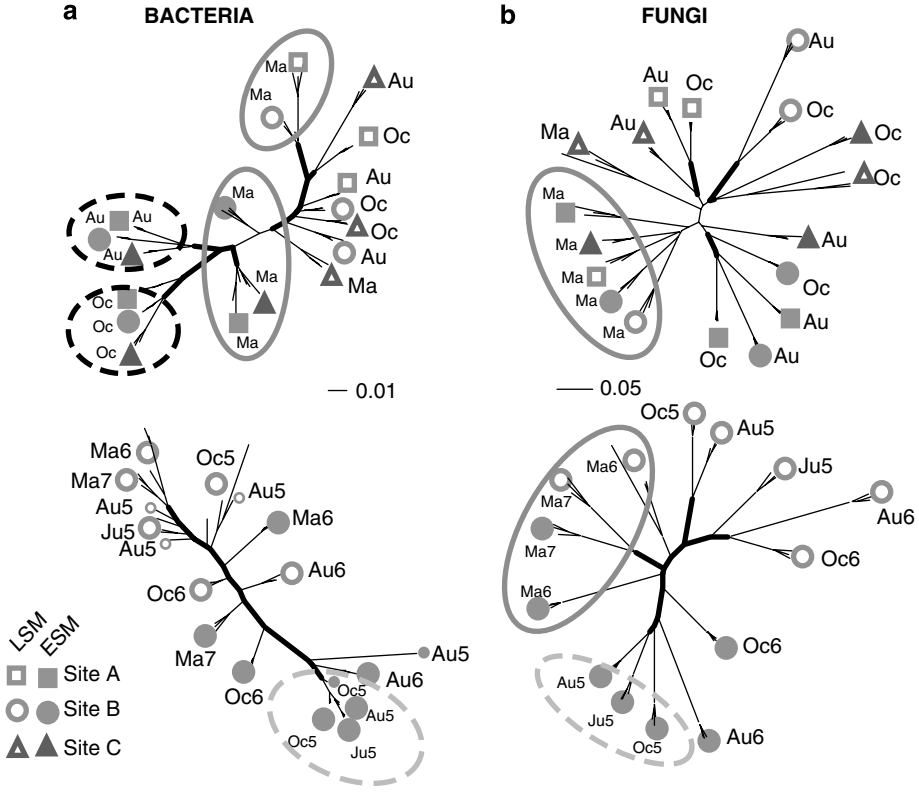

Figure 2 Seasonal variations of bacterial and fungal communities assessed by CE-SSCP. The molecular profile of fungal and bacterial communities was obtained as described in Material and methods, using one DNA pool per each location/site/date. PCRs were carried out by triplicate to limit the influence of PCR biases. Clustering of molecular profiles: (**a**) between three sites from August 2006 to May 2007, (**b**) in the site B during 2 years from June 2005 to May 2007. The ESM locations are in filled symbols and LSM in open symbols, squares indicate Site A, circles Site B, diamond Site C; June, Ju; August; Au; October, Oc; and May, Ma; 2005, 5; 2006, 6; and 2007, 7. Small symbols indicate samples grouping at atypical positions. Molecular fingerprints were compared by computing bootstrapped dendrograms. Thick lines indicate branches supported by a bootstrap value >500. The ovals show the relevant groupings: thick dark-gray lines: May samples post-winter convergence; dark-grey-dashed lines: monthly grouping of ESM sites; light-grey dashed lines: yearly grouping in ESM.

that the observed differences are not because of local conditions but is rather inherent to each location. During the growing season, ESM bacterial fingerprints from the three study sites were consistently grouped according to sampling dates. In contrast, the only grouping for LSM bacteria was found in the May samples. Fungal communities at each location did not display identical seasonal variation for all studied sites. Interestingly, the least distance between microbial communities indigenous to ESM and LSM was observed in May. Although this convergence was strong for fungi, it was less pronounced for bacteria. The same results were found for the three sites, indicating that the shift of microbial communities in May is a general feature of these two habitats. Microbial communities were also followed over 2 years (from June 2005 to May 2007) at one site, always including both studied locations. Similar to data presented in Figure 2a, microbial communities were strongly different between the two locations (Figure 2b). During the growing season, however, microbial communities were not clustered by season, but by year with the exception of May. The aforementioned convergence of microbial communities in May was thus also found to be consistent over 2 years (Figure 2b).

Temporal fluctuations of microbial phylotype composition along the snow cover gradient

Clone libraries were constructed from samples of one site from June 2005 to May 2006. These libraries comprised of small subunit ribosomal DNA (for bacteria), and large subunit ribosomal DNA (for fungi), and consisted in ~350 sequences/library. These sequences were taxonomically assigned using Ribosomal Database Project's Classifier (Cole *et al.*, 2003) for bacteria and BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) for fungi. As illustrated in Figure 3, ESM bacterial clone libraries were dominated by the phyla *Acidobacteria* (22 ± 11%), *Actinobacteria* (18 ± 3%) and *Alphaproteobacteria* (19 ± 4%). In contrast, LSM bacterial sequences were by far dominated by *Acidobacteria* (42 ± 3%) throughout the year, whereas *Actinobacteria* (6% ± 4) were less abundant. For fungi, ESM communities were dominated by *Agaricomycotina* (41 ± 14%), whereas LSM fungal communities

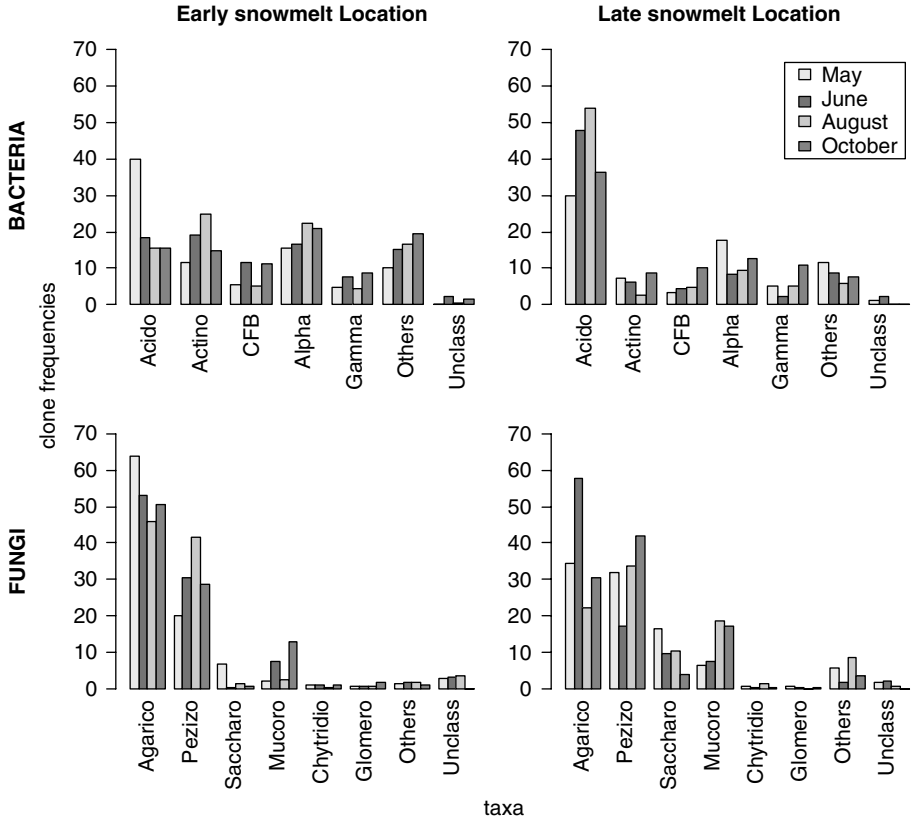

Figure 3 Frequencies of major microbial groups in clone libraries from LSM and ESM. Samples were collected in June, August, October 2005 and May 2006. These libraries consisted of ~350 clones of bacterial 16S rRNA gene or of fungal 28S rRNA gene per sample. For bacteria: Acido, *Acidobacteria*; Actino, *Actinobacteria*; CFB, the *Cytophaga-Flavobacterium* lineage of the *Bacteroidetes*; Alpha and Gamma, α and γ subgroups of *Proteobacteria*; Others: other minor bacterial divisions. For fungi: Basidiomycota are mainly represented by Agarico, *Agaricomycotina*. Ascomycota: Pezizo, *Pezizomycotina*; Saccharo, *Saccharomycotina*. Zygomycota are represented by Mucoro, *Mucoromycotina*; Glomero, *Glomeromycotina*; Chytridio, *Chytridiomycotina*. Others: other minor fungal groups. For the whole figure, Unclass represents unclassified sequences.

appeared more diversified and characterized by the presence of *Saccharomycotina* and *Mucoromycotina*. Furthermore, several seasonal fluctuations in phylotype abundance were observed. For example, *Agaricomycotina* in the LSM location exhibited a sharp increase in June. *Pezizomycotina* was in lower abundance in May in ESM location and in June in LSM location. In October, we noticed an increase of the *Cytophaga-Flavobacterium* lineage of the *Bacteroidetes* (CFB), *Gammaproteobacteria* and *Mucoromycotina* in both locations. In May, we found a noticeable burst of *Acidobacteria* and *Saccharomycotina* and of other minor groups (data not shown) in ESM location that reached the same proportions as those in LSM location.

Temporal and spatial behavior of microbial diversity
The diversity index of microbial communities estimated from clone libraries revealed that bacterial diversity was not different between ESM and LSM locations (Figure 4). Nevertheless, the diversity of ESM bacterial communities was higher in June. In contrast, LSM bacterial diversity was found to be stable throughout all seasons. The diversity of bacterial communities strongly decreased in May, whatever the location. Interestingly, the diversity of fungal communities was noticeably higher in LSM location, particularly in August. In parallel, ESM fungal diversity was found slightly enhanced during June and August. Seasonal variations in fungal and bacterial diversities were thus found to be different between the two studied locations.

Discussion

Alpine tundra is strongly heterogeneous because of the fine-scale variations in topography that lead to differential snow cover. Consequently, plant cover and soil quality in such meadows are highly variable (O'lear and Seastedt, 1994; Litaor *et al.*, 2001; Choler, 2005). Our study locations reflected these variations, having noticeable shifts of snow accumulation (Figure 1a), plant cover, soil organic matter content, soil pH and texture (Table 1, Figure 1b). The results obtained from this study confirmed these trends at the microbiological level. Indeed, the comparison of SSCP profiles revealed pronounced differences between bacterial and fungal communities from ESM and LSM locations, independent of season (Figure 2). These differences were consistent for all study sites (Figure 2a) and throughout the 2-year monitoring (Figure 2b). Thus, these results show a clear distinction between microbial profiles over short distances (5–10 m) at the two extremes of the snow cover gradient, which clearly mirror the surrounding edaphoclimatic conditions as well as the variation in the overlying plant community composition (Table 1, Figure 1).

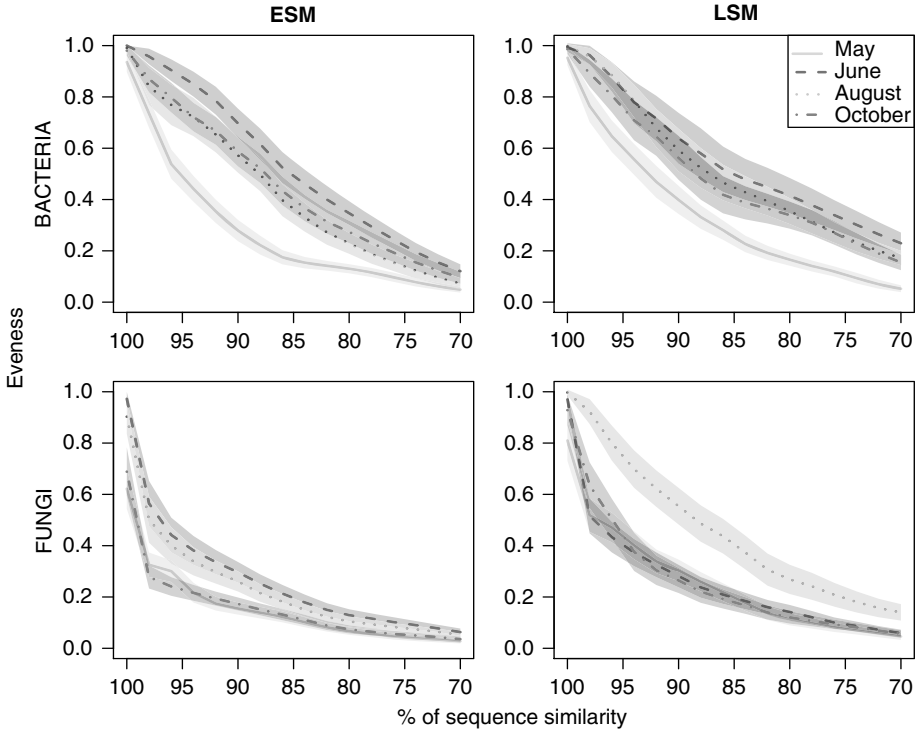

Figure 4 Variations in molecular evenness of bacterial and fungal communities in ESM and LSM, according to the similarity level between sequences. Evenness was estimated from the sequence distance matrix, with 700 resampling by calculating the inverse of the Simpson index and weighted by the library sizes. Each color corresponds to one date sampling and light-coloured areas represent s.d. values. A full colour version of this figure is available at *The ISME Journal* online.

We found that ESM bacterial communities were correlated with growing season progress (Figure 2a). Earlier work indicated that plant cover has a two-fold effect on microbial communities of Antarctic soils. First, when compared with bare soils, the plant cover increases bacterial diversity (Yergeau *et al.*, 2007b). Second, the structure of microbial communities varies with plant cover (Yergeau *et al.*, 2007a). Indeed, root development modifies soil structure, root turnover and litterfall influence carbon input in soil. Moreover, root exudates are composed of various C-containing components (Bais *et al.*, 2006), the quality and quantity of which have been reported to be temporally variable (Farrar *et al.*, 2003). Seasonal variations in bacterial communities may be thus linked to plant development and concomitant rhizodeposition, as shown in greenhouse plants (Butler *et al.*, 2003; Mougel *et al.*, 2006). The bacterial communities of ESM locations seem to be synchronized, probably by the slow growing status of *K. myosuroides* and *D. octopetala*. In contrast, LSM bacteria profiles obtained from August and October samples were separated by small distances (Figure 2a), suggesting the presence of the same phylogenetic groups during the growing season. Conversely, fungal communities either in ESM or in LSM did not show identical seasonal variation whatever the site (Figure 2a), suggesting that the temporal dynamics of these organisms are more sensitive to local, site-specific conditions.

Interestingly, the fewest differences between LSM and ESM microbial communities were always observed in late winter (Figure 2), although this convergence was less pronounced for bacteria. Although seasonal changes in microbial succession have already been described (Schadt *et al.*, 2003; Lipson and Schmidt, 2004; Schmidt *et al.*, 2007; Björk *et al.*, 2008), the convergence of late-winter microbial communities from two contrasting conditions has never been reported. This result suggests that partially identical phylogenetic groups are dominant in both locations at the end of winter. The analysis of the microbial phylotypes shed some light on the basis of this convergence (Figure 3), especially for bacteria. Actually, in late winter, the dominant bacterial phyla in both ESM and LSM are *Acidobacteria* and *Alphaproteobacteria*. Although this convergence was concomitant with soil pH variations and cold temperatures, our data do not allow us to determine what factors are responsible for this winter effect.

These data shown in Figure 2b also provide insights regarding the inter-annual succession of microbial communities. Within each location, microbial communities tended not to be clustered by season, but instead by year, with the exception of winter. The source of this inter-annual variability suggests the existence of a bank of microbial strains in soil represented by only a few individuals (the 'rare biosphere'), similar to the situation that occurs in seawater (Sogin *et al.*, 2006). These yearly changes may arise through the recruitment of functional equivalent strains into the rare biosphere.

The spatial and temporal behavior of microbial communities was further confirmed by DNA sequencing from site B samples (Figure 3), which shed light on ESM and LSM functioning. Bacterial communities in ESM location were dominated throughout the year by the phyla *Acidobacteria* and *Actinobacteria*, which are known for their capacity to degrade recalcitrant substrates (Crawford, 1978; Falcon *et al.*, 1995), as well as by *Alphaproteobacteria* that are often found in rhizosphere (Fierer *et al.*, 2007). In contrast, LSM bacterial communities were by far dominated by *Acidobacteria* throughout the year. This phylum has been found to be well represented in low pH soils (Sait *et al.*, 2006), which may explain their dominance in fairly acidic soils such as in LSM (Figure 1b). Fungal communities in ESM location were dominated by *Agaricomycotina*, with numerous sequences belonging to the genera *Inocybe* and *Russula* (data not shown) (these were earlier reported as *D. octopetala* ectomycorrhiza (Gardes and Dahlberg, 1996)). In contrast, LSM fungal communities appeared more diversified. Thus, in ESM locations, the dominance of symbiotic associations with plants and bacterial species capable of degrading recalcitrant organic matter correlates well with the low fertility observed in ESM locations (Chapman *et al.*, 2006). Environmental conditions seem to promote fungal diversity in LSM location and in *Acidobacteria*. This is possibly caused by higher resource availability (Waldrop *et al.*, 2006) and soil pH (Table 1) for *Acidobacteria* (Lauber *et al.*, 2008).

The phylotype composition of microbial communities was also found to be variable throughout the year, confirming our earlier results (Figure 3). Several microbial groups were indeed found to be linked to growing season (*Alphaproteobacteria*), whereas others, earlier described for their ability to degrade recalcitrant substrates (Cottrell and Kirchman, 2000), emerged during litterfall in ESM as well as in LSM locations (*Gammaproteobacteria*). Late-winter communities were also composed of similar microbial groups.

To further characterize the impact of snow cover patterns on microbes, we estimated bacterial and fungal diversities from clone libraries (Figure 4). Interestingly, bacterial diversity was not influenced by the topographical location. Although bacterial diversity has been described as being strongly influenced by soil pH (Fierer and Jackson, 2006), the range of pH in the studied soils is too small to observe this effect. Bacterial diversity was also found slightly variable across seasons. Nevertheless, ESM bacterial communities were more diversified during the greening phase in June, indicating that plant development promotes bacterial diversity in

such meadows. In agreement with our earlier results, LSM bacterial diversity was stable because of the constant dominance of *Acidobacteria*, implying that this phylum displayed a constant diversity across seasons. However, bacterial diversity dramatically decreased in both locations at the end of winter, suggesting that few bacterial strains are cold tolerant. In contrast, location had a strong impact on the diversity of fungal communities. Fungal diversity was noticeably enhanced in LSM location, particularly at the peak of plant biomass in August. This pattern may be explained by an increase in the nutrient availability due to (i) high root turn over of fast growing plants that are dominant in LSM locations, and (ii) the advanced state of mineralization processes at the end of winter (Bardgett *et al.*, 2005). In contrast, ESM fungal diversity, dominated by ectomycorrhizal fungi, was found lower. Indeed, the ability of this fungal group to switch between saprobic and symbiotic lifestyles (Read and Perez-Moreno, 2003; Martin *et al.*, 2008) may thus allow them to be represented throughout time, independent of resource availability. However, this diversity was enhanced during growing season, possibly in relation to increased root exudation (Bais *et al.*, 2006). These findings highlight different responses between fungal and bacterial diversity along the snow cover gradient.

Conclusion

This paper provides evidence that snow cover dynamics and microbial community composition are strongly interrelated in alpine tundra. Alpine tundra exhibit mosaic of plant communities in relation to fine-scale topographical variations (Körner, 1999). Here, we showed that this strong heterogeneity also occurs at the microbial level. However, further larger-scale surveys are needed to extend this conclusion to other alpine habitats. Moreover, we observed seasonal variations in microbial phylotype composition at each location at the phylum or at sub-phylum levels. This seasonal pattern confirms earlier findings (Schadt *et al.*, 2003; Lipson and Schmidt, 2004; Björk *et al.*, 2008). In contrast to these studies, however, we found that the spatial variations are stronger than the seasonal variations.

This study also reveals that bacterial communities are particularly structured in ESM locations, which show high amplitude of seasonality and limited nutrient availability. In contrast, fungal communities are more stimulated in LSM locations that display weak seasonality and higher nutrient availability. The difference in the response between bacteria and fungi supports the earlier observations of Lauber *et al.* (2008) and may result from their morphological and physiological characteristics, which may be more or less favorable in a given environment (for example, unique cell vs mycelium, enzymatic capabilities, and so on), or from positive or negative interactions between these organisms (Johansson *et al.*, 2004; de Boer *et al.*, 2005; Mille-Lindblom *et al.*, 2006). Moreover, the synchronizing effect of winter can be similar to other extreme events (for example, drought, water logging, and so on). These results may thus be useful to predict microbial successions in the framework of longer time scale studies with varied seasonal or anthropogenic stresses; however, further works are needed to understand the impact of such selective events on ecosystem functioning.

These outcomes also call for a more thorough consideration of snow cover gradients in any attempt to model the carbon cycle of alpine tundra in the context of global change. Considering our results, the change of snow cover dynamics in alpine tundra will have profound impacts on microbial communities. For example, a reduction in snowpack could result in a loss of fungal diversity, as we observed between LSM and ESM locations. Microbes are largely implied in driving large-scale biogeochemical processes. Thus, the question can be raised about the functional importance of the biogeochemical cycles of spatial and seasonal variations of alpine microbial communities, especially under climate change.

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Chapitre II – Article D

Microbial communities assessed by graph partitioning approach: a study of soil Fungi in two alpine ecosystems

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Running Title: Graph partitioning for fungal community studies

ABSTRACT

Understanding microbial community structure and diversity in response to environmental conditions is one of the main challenges in environmental microbiology. However, there is often confusion between determining the phylogenetic structure of microbial communities, and assessing the distribution and diversity of molecular operational taxonomic units (MOTUs) in these communities. This has led to the use of sequences analyses tools such as multiple alignments and hierarchical clustering that are not adapted to large and diverse data sets and not always justified for the MOTUs characterization. Here, we developed an approach combining pairwise alignment algorithm and graph partitioning in order to generate discrete groups in nuclear LSU and ITS1 sequences data sets from a yearly monitoring of two close but contrasting alpine soils, namely early and late snowmelt locations. We tested two clustering methods (Ccomps, single linkage and MCL, Markov clustering) in order to optimize the DNA sequence classification. MCL detected more numerous and cohesive MOTUs, and was thus used to further characterize fungal communities in early and late snowmelt location. We found a few MOTUs specific to one location, but also a contrasting distribution of MOTUs in these two soils, suggesting a high habitat filtering in the community assembly of alpine soil fungi.

1. INTRODUCTION

The assessment of diversity, richness, and composition of microbial communities is a central issue in understanding the effects of environmental conditions on microbial community assembly. In this framework, the massive cloning/sequencing or pyrosequencing of universal DNA markers (*e.g.* rRNA genes) allows characterizing microbial communities in a qualitative and semi-quantitative way (37, 50). The microbial communities composition and diversity based on such methods are assessed (i) by evaluating distances between DNA sequences, (ii) by classifying them in molecular taxonomic units (MOTUs) and (iii) eventually, by identifying MOTUs using reference databases; i.e. the barcoding approaches (48). Although numerous studies focused on the subsequent diversity and richness estimation (24, 39, 44), only a few casted doubt on the alignment methods and sequence classification (25).

First of all, multiple alignment tools are fairly popular in environmental microbiology, although the establishment of phylogenetic links between phylotypes is not necessarily required. Besides, well-suited DNA markers for barcoding approaches require sufficient

variations in composition and size (48), making the analysis of subsequent data sets by multiple alignments difficult. Indeed, based on heuristics, these algorithms excessively penalize insertion/deletion events (32). The pairwise alignments using exact algorithms such as NWS (35), avoid this limitation and have already been applied to bacterial (Hur and Chun, 2004) and fungal communities studies (37, 39). However a great proportion of DNA sequences obtained from massive sequencing are partial due to the interruption of the sequencing reaction, making the NWS algorithm overestimate the distance between complete and partial sequences that are yet similar. The use of a variant of this algorithm, Free end gap (46), presents the advantage to not penalize gap opening or extend in 5' or 3' of the alignment.

The DNA classification is an application of the graph theory in mathematics. It consists in partitioning a graph composed of nodes (here DNA sequences) connected by edges (here similarities) so that each node belongs to only one cluster and is connected with most of the other nodes in this cluster (see Fig. 1). Graph partitioning methods are numerous with different philosophies and the choice of one of them can influence the diversity and richness estimation of microbial communities. The most classical graph partitioning methods, *i.e.* single and complete linkage (3), consist to merge a node or a cluster with the nearest (single link), or the furthest (complete link) neighbours. While the single linkage is a true partitioning method, the latter one relies on maximal clique concept, where one node can belong to several clusters. This feature is often bypassed by artificially organizing the classification into a hierarchy, which is biologically not always justified. On the other hand, single linkage fails to separate clusters connected by one or few nodes, so called chaining effect (see Fig. 1 for an example). The Markov clustering algorithm (MCL) can constitute an alternative to this issue. As single linkage, it is a true partitioning method that additionally simulates the nodes merging according to the local neighbourhood topology, avoiding chaining phenomenon (49). This method is increasingly used in bioinformatics, mostly in proteomic and genetic analyses (7, 13, 29, 52).

Fungi are ubiquitous in soil and able to ensure a large spectrum of functions in ecosystem processes. Involved in nutrient cycling through mycorrhizal associations and litter decomposition, they also strongly effect on plant diversity *via* mutualism or pathogenic interactions (1, 17, 21). Moreover, soils carry a high diversity of fungal species (1, 6, 37) with spatial distribution resulting from many factors such as dispersal, plant coverage, land use and soil properties (14, 27, 30, 43, 51). Temperate alpine tundra landscapes exhibit mesotopographical variations that strongly affect the local snow cover dynamics (28), which

in turn influence soil properties, plant cover and ecosystem processes (10, 12, 31). Alpine tundra thus constitutes a mosaic of ecosystems, distributed along snow cover gradients. Fungi have previously been described as an important component of soil microbial biomass in alpine soils (43) and numerous studies focused on mycorrhizal fungi distribution (11, 18, 34, 41). However, only a few surveys attempted to characterize the whole fungal communities (5, 43). In a previous work, we highlighted co-variations between snow cover dynamics and composition and diversity of soil fungal communities (55). Although these studies gave insights on seasonal succession and diversity of fungal communities in alpine tundra, the assembly of these communities remains poorly characterized.

To avoid the limitations of classical analysis methods for DNA sequence classification, we used pairwise alignments coupled with a graph partitioning approach and tested two partitioning methods, Ccomps (Connected Components) and MCL. This analysis was performed on two data sets resulting from the massive cloning/sequencing of the internal transcript spacer 1 (ITS1) and a part of large subunit rRNA gene (LSU), obtained from two closed soils contrasted by their snow cover duration: Early snowmelt location (ESM) and late snowmelt location (LSM) (see Material and Methods). The most efficient partitioning method was then kept to further characterize ESM and LSM fungal communities and their seasonal variations.

2. MATERIAL AND METHODS

Study site description and sampling: The study site was located in the Grand Galibier massif (French southwestern Alps, 45°.05'N, 06°.38' E, 2480 m elevation). The composition of microbial communities was studied in early snowmelt (ESM) location and late snowmelt (LSM) location. Although these two locations are separated by approximately 20 m, they strongly differ in the duration of winter snow cover because of topographical effects. The shallow winter snowpack in ESM plot leads to soil freezing in winter, and the dominance of a stress tolerant graminoid, *Kobresia muysoroides* (Cyperaceae) and a dwarf shrub, *Dryas octopetala* (Rosaceae). In contrast, LSM location displays a persistent and deep winter snowpack, insulating soil from cold winter temperatures. It is dominated by low-stature species which can support shorter growing season such as *Carex foetida* (Cyperaceae), *Alpecurus alpinus* (Poaceae), *Alchemilla pentaphyllea* (Rosaceae) and *Salix herbaceae* (Salicaceae) (4, 10). The soils of these two plots also differ in their clay content, higher in LSM, and by their organic matter content, higher in ESM. Soil pH ranges from ~5.5 in LSM to ~6.5 in ESM, but this feature is inverted in May (55).

Four sampling dates were chosen to follow up variations of microbial communities in relation with snow cover dynamics: June 24th 2005 when the growing season started in ESM plot while the snow melted in LSM plot, August 10th 2005 during the peak of standing biomass, October 10th 2005 during litter fall, and May 03rd 2006, at the end of winter, just after thawing in ESM plot while LSM plot was still under a consistent snowpack of 2.5 m depth. Five spatial replicates were sampled in each plot at each date, and sieved at 2 mm in order to homogenize soils and remove rocks and the main part of roots.

Clone library construction: Soil DNA extraction was carried out with the Power Soil Extraction Kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) as previously described (Zinger et al., 2009) and the DNA extracts from the five spatial replicates were pooled to limit the soil spatial heterogeneity according to Schwarzenbach et al. (45). From the 8 remaining DNA pools, the LSU gene was amplified with the primers U1 (5'-GTGAAATTGTTGAAAGGGAA-3') (42) and nLSU1221R (5'-CTAGATGAACYAACACCTT-3') (43) as described in (55). A second clone library of ITS1 region was also constructed with the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAACG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (54) with the same PCR conditions reported in (Zinger et al., 2009) for CE-SSCP (Capillary Electrophoresis Single Strand Conformation Polymorphism). For both LSU and ITS1, eight independent PCR-amplifications were performed and then mixed for each DNA pools in order to dilute PCR biases.

The 16 PCR-products were then cloned using TOPO TA PCR 2.1 cloning kit (Invitrogen SARL, Molecular Probes, Cergy Pontoise, France) and ligations were sent to the Centre National de Sequençage (Genoscope, Evry, France) for transformation and sequencing. A total of 2844 and 2956 sequences were obtained for LSU and ITS1 respectively, ranging from 350 to 380 sequences per sample.

Genbank accessions are FJ568339 to FJ570564 for LSU sequences (55), and FN293398 to FN295479 for ITS1 (this study).

Sequence analysis. DNA sequences obtained from the 16 clone libraries were grouped in one data set for each DNA marker. Sequences smaller than 100 bp (ITS1) or 500 bp (LSU) were removed from the analysis to ensure the overlap of all sequences. Our data sets contained numerous sequences variable in length (incomplete LSU sequences, high size polymorphism of ITS sequences), preventing to obtain reliable alignments by using multiple alignment tools. We thus performed pairwise alignments based on the Free End gap dynamic

programming alignment algorithm (46) and computed similarity matrices using the software 'borneo' (available under request at eric.coissac@inrialpes.fr).

Based on these similarity matrices, both graphs were submitted to a single linkage method, Ccomps (www.graphviz.org) and to the Markov clustering algorithm MCL (micans.org, [49]), for 95, 97 and 98% of similarity thresholds. The main parameter of MCL, inflation, also called granularity, determines the partition sharpness. The parameters of MCL were adjusted as follow: initial inflation of 6 and main iteration of 100. The graph visualisation was obtained by using fdp (www.graphviz.org). The clusters cohesion for Ccomps and MCL was assessed for each cluster as follow: $(e/E) \times (n/N)$, where e is the count of edges observed, E the maximum count of edges, n the count of nodes, and N the total count of nodes in the graph. This will return to a value between 0 and 1, 0 meaning that none nodes are connected in the graph, 1 meaning that all nodes are connected together.

Each DNA sequence was then compared with imported references from GenBank database (http://www.ncbi.nlm.nih.gov) in November 2008, using BLAST (2). The best blast match was kept for sequence identification. Non-matching or partially matching sequences were considered as non-exploitable and thus discarded for subsequent analyses. Because of the ambiguity of the rarest MOTUs (*i.e.* containing less than 5 sequences), they were not considered in the analysis of ESM and LSM fungal communities. Based on BLAST results, the congruence of the taxonomical information carried by LSU and ITS1 markers was checked at the order level, by comparing the abundance of fungal orders detected in both data set using the non-parametric Kendall tau rank correlation test. This test was also used to compare the MOTUs distribution between ESM and LSM fungal communities. Statistical analyses were performed with the R software (40).

3. RESULTS

Comparison of Ccomps and MCL clustering methods

Based on similarity matrices obtained from pairwise alignments, the two clustering methods did not result in the same discretization of MOTUs, as illustrated in Fig. 1 for a sub-data set. Indeed, MCL generated more clusters than Ccomps, which was particularly noticeable for the LSU graph (Table 1): while MCL increased ~1.2 fold the number of MOTUs in ITS1 graph, it resulted in 2 to 8 fold more LSU-MOTUs. In the same way, the number of singletons obtained from MCL was found almost constant while they were found

less numerous and increased with similarity thresholds by using Ccomps (Table 1). Finally, the cohesion of MOTUs, *i.e.* the number of edges within one MOTU, was found higher for MOTUs obtained by MCL (Table 1). Here again, the use of MCL increased the MOTUs cohesion ~15 fold for ITS1 data set while it resulted in 14 to ~2500 fold more cohesive MOTUs for LSU data set. The multiple alignments of each clusters obtained from MCL were found consistent (data not shown).

Table 1: Comparison of LSU and ITS1 graphs partition by Ccomps and MCL according to different similarity thresholds.

DNA region	Clustering method	Similarity threshold (%)	Clustering performances		
			No. groups[a]	No. singletons[a]	Cohesion index[b]
LSU	Ccomps	95	11	23	17.23 ± 32.71 *(5)*
		97	34	63	3.09 ± 7.37 *(27)*
		98	56	115	0.09 ± 0.16 *(36)*
	MCL	95	56	124	244.59 ± 97.04 *(25)*
		97	111	133	215.44 ± 115.89 *(48)*
		98	125	133	248.13 ± 130.65 *(62)*
ITS1	Ccomps	95	153	185	8.67 ± 16.46*(78)*
		97	191	227	9.16 ± 16.11 *(89)*
		98	203	274	8.82 ± 15.57 *(96)*
	MCL	95	182	185	142.61 ± 65.25 *(93)*
		97	207	227	117.70 ± 54.40 *(97)*
		98	214	277	127.77 ± 130.65 *(100)*

[a] Based on the total data sets (n=2661 for LSU, 2956 for ITS)
[b] Values × 10^{-3} of cohesion index correspond to mean ± SD *(No. of MOTUs)*. Based on MOTUs containing more than 4 sequences.

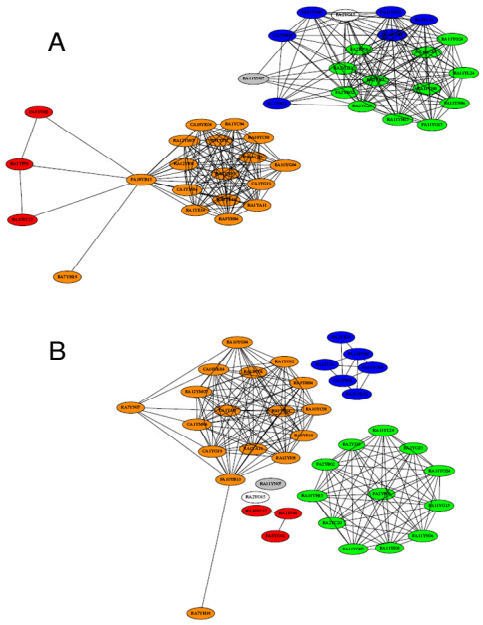

Figure 1: Weighted non-directed graph showing the clustering results of a subdataset submitted to (A): Ccomps, and (B): MCL clustering. Each node represents a clone and each edge represents a similarity of at least 98 % between two clones. The colours of labels illustrate the subgroups formed by Ccomps and MCL. The graphic was drawn with fdp (www.graphviz.org).

Distribution of MOTUs between samples

For this analysis, we kept the MOTUs composed of at least five sequences (see Material and Methods). For a threshold of 98%, MCL resulted in 62 and 100 MOTUs for LSU and ITS1 respectively. The taxonomic identification of DNA sequences performed by BLAST revealed only one LSU-MOTU (17 clones) and three ITS1-MOTUs (27 clones in total) corresponding to non fungal sequences (stramenophiles or plants). Also, 18 MOTUs of ITS1 dataset, corresponding to 227 clones, which were suspected as chimera or didn't match against Genbank references, were removed for the subsequent analyses. The remaining MOTUs consisted in 60 for LSU and 79 for ITS1. At the genus level, while most of ITS1-sequences displayed the same identity within each MOTU, it was less consistent for LSU data set (supplementary Tables S1 and S2). The congruence of MOTUs repartition according to the DNA marker used was then checked by comparing the rank of fungal orders (*sensu* taxonomy) abundance that were common to both data set (Fig. 2A). This resulted in a positive correlation supported by the Kendall tau rank correlation test ($\tau = 0.723$ and $p < 0.001$ for ESM and $\tau = 0.566$ and $p < 0.01$ for LSM), which is mostly due to the dominant taxonomic groups.

Most of MOTUs displayed a contrasting distribution among locations (Fig. 2B) based on Kendall tau rank correlation test: $\tau = - 0.202$ $p < 0.05$ for LSU, $\tau = - 0.399$ and $p < 0.001$ for ITS1. However, only 9 of LSU-MOTUs were found strictly specific to ESM location and 16 to LSM location. In the same way, 21 ITS1-MOTUs were found specific to ESM location against 17 to LSM one. These specific-location MOTUs were rare most of the time. On the other hand, few MOTUs were found equally represented in both locations (Fig. 2B). Because almost 80% of clones were found distributed in thirty MOTUs, whatever the samples and the DNA marker used, we focused on these MOTUs for a sharper description

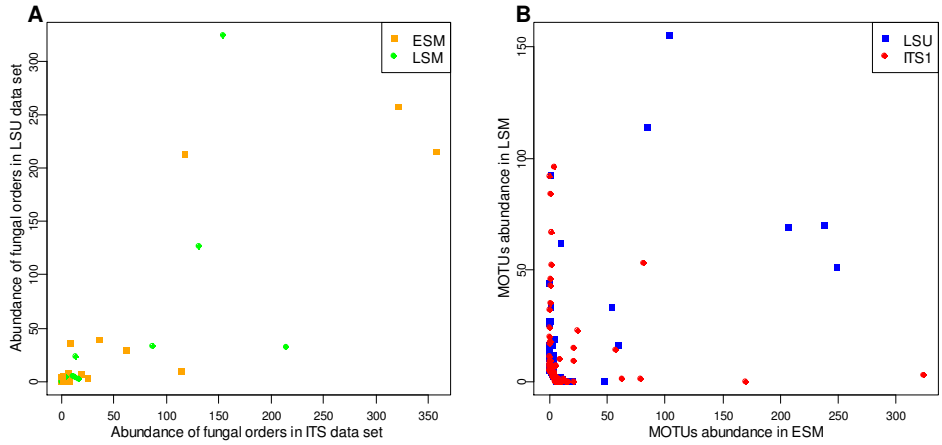

Figure 2: Comparison of MOTUs abundance according to the DNA markers and the studied locations. A/ Abundance of MOTUs belonging to common fungal orders between the LSU and ITS1 markers are compared. B/The abundance of MOTUs are compared between LSM and ESM. The non-parametric statistic Kendall tau rank correlation coefficient displayed a significant correspondence degree between the abundance of fungal orders and the DNA marker used, and a negative correspondence degree between MOTUs abundance between the two studied locations.

Spatial and seasonal variation of abundant MOTUs

LSU data set: Globally, ESM location displayed few dominant and a majority of rare MOTUs except in May, where they were more uniformly distributed. Similarly to what occur in ESM-May sample, MOTUs displayed a uniform distribution in LSM location (Fig. 3). Nevertheless, this location was noticeably dominated by L3 (*Helotiales*) and L5 (*Mortierellaceae*) in August and October and by the group L23 (*Tricholomataceae*) in June. In ESM location, *Basidiomycota* were found meanly more abundant, mainly represented by the MOTUs L1 (*Cortinariaceae*), L4 (*Cortinariaceae* and *Russulaceae*), L9 and L10 (*Clavulinaceae*), but also by the group L3 (*Pleosporale, Ascomycota)*. These MOTUs were found mostly stable during the growing season, except for *Clavulinaceae*-MOTUs, which appeared overrepresented in May.

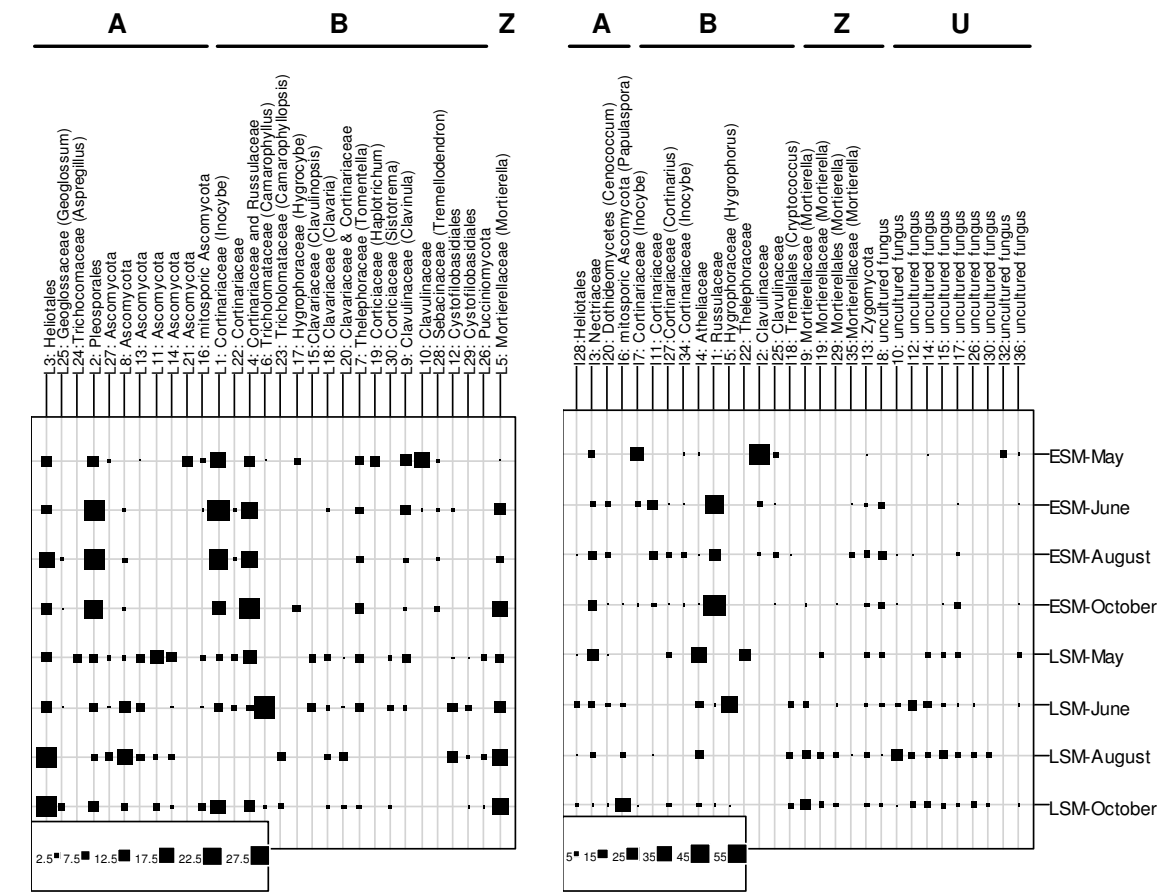

Figure 3: Spatial and temporal distribution of the main MOTUs. A: LSU data set, B: ITS data set. The columns of diagram represent the thirty most abundant MOTUs. Lines of the diagram represent samples. Square size indicates de percentage of clones in each sample. A: Ascomycota, B: Basidiomycota, Z: Zygomycota, U: Unclassified fungi.

ITS data set: Ascomycota were much less represented in ITS1 data set (Fig. 3). As for LSU data set, MOTUs distribution was more uniform in LSM location. However, several MOTUs displayed a punctual dominance, namely, I3 (*Hypocreales*) in May and I6 (mitosporic *Ascomycota*) in October, and the MOTUs I4 (*Atheliaceae*) and I22 (*Thelephoraceae*) in May, as well as I5 (*Hygrophoraceae*) in June. Unfortunately, a large part of LSM-MOTUs are not identified. ESM location was largely dominated by *Basidiomycota*, particularly by the MOTUs I1 (*Russulaceae*) from June to October, and by I7 (*Cortinariaceae*) and I2 (*Clavulinaceae*) in May.

4. DISCUSSION

DNA analyses considerations

DNA sequences can be seen as a continuum where each sequences shares more or less similarities with other ones. To assess these similarities, alignment is a critical step and numerous and varied tools exist for this purpose. While multiple alignments can answer to phylogenetic questions, they are not appropriate for the assessment of diversity and structure of microbial communities based on DNA barcoding approaches. In this context, the Free End Gap algorithm is much more appropriate because it is less affected by insertion/deletion events and do not penalize partial DNA sequences, a current feature in large datasets. The comparison of microbial communities in such continua is possible by evaluating the DNA sequence similarity intra-community *vs*. inter-communities (53). However, this comparison remains poorly informative about microbial communities themselves. In contrast, an artificial discretization of this continuum allows an easier comparison of microbial communities by producing data comparable to the traditional taxonomy-based approaches. In this context, the DNA classification is usually assessed through hierarchical clustering methods (44). However, this approach is not justified given that connexion between DNA classes are not necessarily required for a global characterization of microbial communities. In this context, a simple discretization of our continuum is sufficient, and can be assessed by graph partitioning methods.

A graph partitioning method should ideally be able to (i) produce highly cohesive and discrete classes, (ii) detect singletons, (iii) be efficient in time computation to process large datasets. The conservative DNA regions, such as LSU region, generate more continuum structures than less conserved region, such as ITS1. Graphs containing large continuum structures will likely be harder to partition. Indeed, the partition of the ITS1 graph resulted in more MOTUs by both clustering methods (Table 1). This feature is due to chaining effects that merge poorly connected entities (illustrated in Fig. 1). A good partitioning method should thus also be able to form discrete classes within these continuum structures. The MCL algorithm has been previously reported as highly efficient to process large datasets in a more robust way (7). Our observations support the performance of MCL, which produced more numerous and cohesive MOTUs than Ccomps, especially for the LSU graph (Table 1, Fig. 1). This result reflects the ability of MCL to avoid chaining effects in continuum structures. Furthermore, it points out that the clustering methods can generate erroneous results, as the small number of LSU-MOTUs found by Ccomps. In the same way, MCL detected a

maximum number of singletons since the lowest similarity thresholds while Ccomps did not reach this maximum at 98% of similarity (Table 1). These results show that the choice of clustering methods is determinant in the assessment richness and diversity, and that MCL is more appropriated for our purpose. Besides, MCL could be used for other conservative DNA regions such as 16S rDNA for analysing prokaryotic communities. Although this method has already been used in comparative genomic analyses (29, 52) it is the first time to our knowledge that this algorithm is applied to the characterization of microbial communities.

Despite the seizure of discrete and cohesive clusters, the taxonomic assignation of MOTUs, especially for LSU data set (Table S1), remained problematic for three main reasons. First of all, a "universal similarity threshold" does not exist and varies according to the taxonomic family considered and the DNA marker used (6). Second, the taxonomy of fungi is still evolving and can hardly be fixed because of the paraphyletism or uncertainty of phylogenetic position of certain fungal groups such as *Helotiales* (23) and *Russulales* (22). Third, the quality of public databases is doubtful: 20% of fungal sequences in international databases may be incorrect at the species level (36). For instance, *Cryptococcus* Genbank sequences (*Tremellales, Basidiomycota*) are annotated as belonging to *Tremellales* but also to *Cystofilobasidiales*, *Filobasidiales*, and to *Ascomycota* phylum. Although the abundance fungal orders were found quite similar between LSU and ITS1 data sets (Fig. 2A), the use of taxonomy as criteria of DNA classification can be casted doubt in this framework, and should thus rather be based on the sequences similarities. Moreover, the taxonomic assignation of MOTUs and subsequent ecological conclusions should be made carefully.

The use of graph partition approach based on pairwise alignment and coupled with MCL clustering thus seems a good alternative to analyse large DNA sequence datasets obtained from pyrosequencing technique, avoiding the recurrent issues of "manual corrections" of multiple alignments and the references databases errors. Although this method does not explain the evolutionary connexion between MOTUs, it allows the assessment of microbial community structure, richness and diversity and may also constitute a first filtration step to create more homogeneous sub-data sets for further phylogenetic studies, as suggested by Loytynoja & Goldman (32).

Phylotype diversity in ESM and LSM soils

The fungal communities of both locations displayed few dominant and numerous rare MOTUs (Fig. 2B). For instance, the ten more abundant MOTUs accounted from 40 to 90% of each clone libraries that typically reflects what is observed in macro-organisms communities

(20, 24). Interestingly, ESM location displayed few highly dominant MOTUs compared to LSM location, supporting our previous findings (55) relating the higher diversity of fungal communities in LSM location.

Although our studied locations are spatially close, the contrast in snow cover dynamics, vegetation type, soil properties, and ecosystem processes are strong (F. Baptist, G. Yoccoz and P. Choler, submitted for publication) and co-varies with microbial communities (55). These differences significantly affect the distribution of MOTUs whatever the DNA marker used (Fig 2B, Fig. 3). However, only few MOTUs were found strictly specific to one location or one season and the differences of fungal communities between the two locations were rather expressed in terms of abundance (Fig. 3). Most of these site-specific MOTUs were rare, and our sequencing effort may have been insufficient to detect them in the other location. The lack of MOTUs specificity to one site can be explain by the high dispersal rate of these organisms (16) and the short distance between the studied locations, as observed by (15) for freshwater bacterial communities.

On the other hand, the abundant MOTUs in LSM location were mostly rare in ESM location and *vice-versa* (Fig. 2B, Fig. 3), suggesting that contrasted environmental conditions filters contrasted fungal communities. Indeed, ESM correspond to stressful and nutrient limited habitat (10). This location was dominated by phylotypes related to ectomychorrizal fungi (*e.g. Cortinariaceae*, *Russulaceae*, *Helotiales*; Fig. 3) often associated with the ESM-dominant plants *Kobresia myosuroides* and *Dryas octopetala* (18, 34, 43); and to *Pleosporales*, previously described as dark septate fungi strongly involved in nitrogen translocation in alpine grasslands(19, 38). These dominance patterns were stable from spring to autumn suggesting that these fungi shift along a parasitism/mutualism continuum trough both saprotrophic activites and nutrient uptake capacities (26, 33). In late-winter, these fungal groups declined in aid of presumably psychrotolerant *Cantharellales* (Fig. 3); known to form ectomycorrhizal associations (47) and for lignin degradation abilities (9). The dominance of ectomycorrhizal associations and phylotypes related to fungal species able to degrade complex organic matter returns to what occur in ecosystems displaying slow nutrient turnover (8), such as ESM location. In contrast, LSM location displays fast litter degradation rate and high nutrient availability (F. Baptist, G. Yoccoz and P. Choler, submitted for publication). LSM-fungal communities appeared more temporally variable, and more diversified with an important part of poorly documented fungal groups, precluding further conclusions (Fig. 3). However, this higher diversity is potentially related to a higher nutrient availability (51) and has been associated to open nutrient cycle ecosystems (8).

5. CONCLUSION

The recent development of high-throughput DNA sequencing technologies opens new avenues in environmental microbiology. This requires improvement in the way to process such amazing large datasets, from alignments to diversity estimators. Here, we show that the use of pairwise alignment coupled with the fast processing and efficient MCL clustering is a reliable technique to analyse large datasets for microbial communities' studies. It also constitutes a first step for further phylogenetic analyses.

Applied to the soil fungal communities of two contrasted habitats, this analysis pathway confirmed the importance of habitat filtering in the assembly of fungal communities (55). This study constitutes a first step towards the functioning of these two contrasted ecosystems. Further characterisations via culture or microscopic approaches coupled with meta-transcriptomics will be helpful to determine the mycorrhizal, pathogen or saprophytic status of MOTUs described here. Finally, the linkage between our findings and those of other microbial groups, such as bacteria (B. Shahanavaz, L. Zinger, S. Lavergne, P. Choler and R.A. Geremia, to be submitted) will provide a more comprehensive of microbial dynamics in these ecosystems.

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SUPPLEMENTARY MATERIAL

Table S1: Summary of best blast match within the main LSU-MOTUs defined by MCL

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
1	AY380383	*Inocybe leiocephala*	117	98.75	718.35
1	AY380384	*Inocybe leptocystis*	44	97.73	707.27
1	AY239021	*Inocybe chelanensis*	39	98.77	779.69
1	EU600831	*Inocybe sp.BK 21089718*	28	98.84	739.57
1	EU600863	*Inocybe sororia*	14	99.02	700.29
1	DQ071697	*Inocybe rimosa*	13	99.53	770.85
1	AY380378	*Inocybe griseolilacina*	9	97.87	735.33
1	EU307845	*Inocybe subexilis*	5	99.16	689.40
1	EU600893	*Inocybe sp.BK 27089703*	5	98.24	715.60
1	AY239019	*Inocybe candidipes*	4	97.51	712.75
1	EU307837	*Inocybe sp.SA1 00602A*	3	99.38	809.67
1	AY380375	*Inocybe flocculosa*	3	97.46	699.67
1	AY038316	*Inocybe godeyi*	3	96.67	743.00
1	EU569858	*Mallocybe leucoblema*	2	99.85	684.50
1	AY038318	*Inocybe lacera*	2	98.44	766.50
1	DQ071740	*Flammulaster muricata*	2	97.77	695.50
1	EU600895	*Inocybe sp. GDa*	2	96.12	781.00
1	EU569852	*Inocybe sp. DED8050*	2	95.54	717.00
1	AY380394	*Mallocybe sp. PBM 2397*	1	99.59	734.00
1	AY380367	*Inocybe armeniaca*	1	99.18	607.00
1	EU555444	*Inocybe althoffiae*	1	98.83	768.00
1	EU307825	*Inocybe cf.hirtella PBM 2619*	1	98.53	612.00
1	AY380391	*Inocybe serotina*	1	97.70	740.00
1	EU307827	*Inocybe bakeri*	1	97.09	790.00
1	AY380370	*Inocybe cerasphora*	1	96.67	810.00
1	AY380360	*Cortinarius aureifolius*	1	96.45	789.00
1	EU307822	*Inocybe hirtella*	1	96.31	813.00
1	EU569843	*Inocybe sp. PBM 2132*	1	94.37	568.00
2	DQ384107	*Neotestudina rosatii*	160	95.09	725.86
2	DQ384104	*Zopfia rhizophila*	47	95.50	723.83
2	DQ678071	*Herpotrichia diffusa*	20	97.22	732.40
2	DQ678078	*Pleomassaria siparia*	16	98.20	641.31
2	DQ678080	*Herpotrichia juniperi*	11	98.76	684.18
2	DQ678067	*Lepidosphaeria nicotiae*	8	95.74	696.25
2	DQ678081	*Lophium mytilinum*	6	96.30	691.00
2	DQ678076	*Ulospora bilgramii*	6	96.09	693.67
2	AY544686	*Preussia terricola*	5	98.16	566.00
2	AY436405	*Hilberina caudata*	3	98.40	746.67
2	AY016357	*Byssothecium circinans*	3	98.04	728.33
2	DQ678061	*Cucurbitaria elongata*	3	97.54	774.33
2	DQ384094	*Lophiostoma macrostomum*	2	98.88	585.00
2	DQ678072	*Trematosphaeria pertusa*	2	97.43	621.50
2	DQ678051	*Botryosphaeria dothidea*	2	95.35	698.50
2	DQ384092	*Leptosphaeria macrospora*	1	100.00	769.00

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
2	DQ384098	*Sporormia lignicola*	1	98.97	776.00
2	DQ678056	*Preussia minima*	1	98.58	777.00
2	EU223257	*Phaeosphaeria avenaria*	1	97.61	712.00
2	DQ986799	*Umbilicaria aprina*	1	95.92	710.00
2	EF445987	*Drechslerella brochopaga*	1	93.98	781.00
3	AY064705	*Neofabraea alba*	68	98.06	707.76
3	AY789415	*Hyaloscypha daedaleae*	46	98.11	743.52
3	AY544680	*Crinula caliciiformis*	41	98.15	707.54
3	DQ470967	*Pezicula carpinea*	33	97.48	689.15
3	DQ227263	*Hyphodiscus hymeniophilus*	19	98.47	701.00
3	DQ470988	*Pseudeurotium zonatum*	15	97.40	762.60
3	EU883432	*Tetracladium furcatum*	14	98.79	668.14
3	AY544646	*Lachnum virgineum*	5	99.05	760.00
3	EU883431	*Tetracladium breve*	5	98.48	723.40
3	DQ470944	*Cudoniella clavus*	4	97.25	686.25
3	AF356696	*Rhytisma acerinum*	3	97.36	669.00
3	AY544656	*Chloroscypha cf.enterochromaOSC100020*	2	100.00	272.00
3	AY544651	*Botryotinia fuckeliana*	2	99.59	708.00
3	AY789431	*Hymenoscyphus scutula*	1	98.72	549.00
3	AY640973	*Thelocarpon laureri*	1	93.94	594.00
4	AF506462	*Russula nauseosa*	163	98.14	741.94
4	AY380360	*Cortinarius aureifolius*	68	97.01	752.25
4	DQ986294	*Pachylepyrium carbonicola*	18	98.01	689.78
4	AY380405	*Naucoria escharoides*	8	98.47	711.25
4	AY631899	*Lactarius deceptivus*	5	94.85	781.00
4	AF506411	*Lactarius leonis*	4	96.67	809.75
4	EF561632	*Hebeloma affine*	2	99.63	721.00
4	AY380361	*Cortinarius fibrillosus*	2	96.72	752.50
4	AY745703	*Hebeloma velutipes*	1	99.55	665.00
4	AF506433	*Gloeocystidiellum aculeatum*	1	99.12	567.00
4	AF506465	*Russula violacea*	1	97.29	739.00
4	DQ071740	*Flammulaster muricata*	1	95.60	682.00
4	AY544680	*Crinula caliciiformis*	1	92.02	639.00
5	DQ273794	*Mortierella verticillata*	181	97.83	696.13
5	EU688966	*Mortierella indohii*	13	97.03	752.23
5	AY586632	*Athelia decipiens*	1	98.30	589.00
5	DQ973031	*Masonhalea richardsonii*	1	95.04	685.00
5	DQ518999	*Zygozyma smithiae*	1	94.01	334.00
5	EU569843	*Inocybe sp.PBM2132*	1	93.87	587.00
5	DQ986799	*Umbilicaria aprina*	1	93.25	711.00
6	DQ457650	*Camarophyllus aff.pratensisPBM2752*	92	97.63	731.76
6	DQ071740	*Flammulaster muricata*	1	94.91	805.00
7	DQ835997	*Tomentella sp.AFTOL-ID1016*	87	98.05	744.02
8	DQ986798	*Bacidina arnoldiana*	25	92.51	485.76
8	EU011612	*Pichia finlandica*	22	92.57	563.77
8	EU011630	*Pichia pilisensis*	9	96.37	428.00
8	AY548296	*Saitoella complicata*	9	92.19	574.44
8	EF550329	*Pichia bimundalis*	4	92.18	594.00
8	EU011634	*Candida ovalis*	1	92.57	565.00
8	AY533015	*Geoglossum glabrum*	1	91.34	543.00

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
8	DQ273799	*Lecophagus sp.ATCC56071*	1	90.29	577.00
9	AM259212	*Clavulina cristata*	75	99.59	746.24
10	AJ534892	*Clavulinaceae sp. F46*	48	96.57	756.63
11	EF550301	*Candida sp. NRRLYB 2243*	35	92.13	557.03
11	DQ986798	*Bacidina arnoldiana*	5	91.99	447.00
11	EU011630	*Pichia pilisensis*	3	95.33	428.00
11	EF550285	*Candida sp. NRRLY 17713*	1	91.67	540.00
12	DQ667161	*Itersonilia perplexans*	29	95.12	560.90
12	DQ832234	*Sporobolomyces roseus*	2	95.10	510.00
12	DQ836005	*Udeniomyces puniceus*	2	94.69	573.50
12	DQ831016	*Mrakia frigida*	1	92.57	417.00
13	EU011615	*Candida sp. NRRLYB 2442*	22	95.51	269.82
13	DQ986798	*Bacidina arnoldiana*	5	93.74	322.40
13	EU011612	*Pichia finlandica*	1	92.55	564.00
14	EU011615	*Candida sp. NRRLYB 2442*	24	95.45	269.83
14	AY745727	*Bensingtonia yuccicola*	3	91.61	298.00
15	EF535269	*Clavulinopsis subtilis*	23	93.03	741.74
15	EF535273	*Clavulinopsis fusiformis*	3	97.54	595.00
16	EU883432	*Tetracladium furcatum*	16	95.78	717.13
16	DQ470967	*Pezicula carpinea*	3	97.30	652.00
16	AY789415	*Hyaloscypha daedaleae*	3	97.10	712.00
16	DQ470944	*Cudoniella clavus*	1	97.42	271.00
16	AY544680	*Crinula caliciiformis*	1	94.38	640.00
17	AY684167	*Hygrocybe aff. conica PBM918*	20	98.58	777.40
18	AY463395	*Clavaria argillacea*	18	97.17	749.83
19	EU118629	*Haplotrichum curtisii*	20	95.98	405.15
20	AY586646	*Clavaria fumosa*	6	92.84	633.00
20	EU555447	*Inocybe sp. E5563*	3	93.79	365.00
20	EU569853	*Inocybe glaucodisca*	3	93.37	452.67
20	AY646099	*Insolibasidium deformans*	1	95.47	419.00
20	EU600852	*Inocybe rimosa*	1	92.23	566.00
21	EU011615	*Candida sp. NRRLYB 2442*	12	96.30	270.00
21	DQ986798	*Bacidina arnoldiana*	6	92.96	324.17
22	AY380408	*Flammulaster sp. PBM 1871*	8	96.88	759.25
22	DQ457650	*Camarophyllus aff. pratensis PBM 2752*	5	99.14	594.80
22	DQ071700	*Phaeomarasmius rimulincola*	2	97.33	729.00
22	DQ457668	*Galerina atkinsoniana*	1	95.54	717.00
23	EF561628	*Camarophyllopsis hymenocephala*	8	94.06	609.88
23	AF506387	*Dentipellis fragilis*	5	93.30	397.40
24	AM270052	*Aspergillus niger*	15	92.45	728.20
24	EF626957	*Eupenicillium ochrosalmoneum*	1	93.48	782.00
25	AY544650	*Geoglossum nigritum*	11	98.08	714.64
25	AY533015	*Geoglossum glabrum*	3	98.12	698.67
25	AY789428	*Sarcoleotia globosa*	1	97.46	709.00
26	AY745727	*Bensingtonia yuccicola*	12	91.40	294.83
27	AY584641	*Xanthoparmelia conspersa*	6	94.96	620.67
27	AY640973	*Thelocarpon laureri*	6	93.39	683.50
27	DQ470961	*Rhizina undulata*	1	93.38	423.00
27	AF329171	*Ochrolechia balcanica*	1	93.10	710.00

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
28	AY745701	*Tremellodendron sp. PBM 2324*	13	98.17	766.38
28	DQ521406	*Sebacina incrustans*	1	98.86	699.00
29	DQ836005	*Udeniomyces puniceus*	8	94.76	495.13
29	DQ667161	*Itersonilia perplexans*	2	94.60	601.00
30	AM259215	*Sistotrema coroniferum*	12	96.21	735.00
30	AM259214	*Multiclavula vernalis*	1	97.65	638.00
31	EU011615	*Candida sp.NRRLYB-2442*	6	95.13	270.00
31	DQ986798	*Bacidina arnoldiana*	6	93.40	323.00
33	DQ457683	*Inocephalus sp.GD-b*	5	96.70	753.40
33	AF261580	*Pluteus umbrosus*	4	97.70	715.75
33	AY745710	*Volvariella gloiocephala*	1	98.84	606.00
33	AY700180	*Entoloma prunuloides*	1	96.80	657.00
33	AY691891	*Entoloma sinuatum*	1	96.56	784.00
34	AF279413	*Stereocaulon paschale*	5	97.03	772.80
34	EF420058	*fungal endophyte*	3	97.20	594.00
34	DQ678091	*Cercospora beticola*	2	98.86	743.00
34	DQ986799	*Umbilicaria aprina*	2	98.46	490.50
35	AY691807	*Bolbitius vitellinus*	6	93.28	681.83
35	AY038329	*Phaeomarasmius curcuma*	5	91.26	746.20
35	EF535272	*Phaeomarasmius proximans*	1	95.09	611.00
36	DQ273794	*Mortierella verticillata*	8	95.00	709.75
37	AY544661	*Cheilymenia stercorea*	10	98.04	746.10
38	AY789428	*Sarcoleotia globosa*	7	95.03	720.86
38	DQ273799	*Lecophagus sp.ATCC56071*	2	95.98	715.50
38	AY544650	*Geoglossum nigritum*	1	96.99	531.00
38	EF445989	*Orbilia auricolor*	1	95.97	720.00
39	DQ470967	*Pezicula carpinea*	8	95.77	758.00
39	DQ227263	*Hyphodiscus hymeniophilus*	3	96.95	675.00
39	AY544683	*Monilinia fructicola*	1	97.68	517.00
40	AY586646	*Clavaria fumosa*	6	93.21	593.17
40	AY463395	*Clavaria argillacea*	1	93.06	605.00
41	EF535264	*Hygrophorus purpureofolius*	11	98.18	603.55
42	DQ071712	*Lepiota xanthophylla*	6	98.92	751.00
42	DQ071709	*Bovista nigrescens*	4	98.93	646.00
42	DQ911601	*Leucoagaricus barssii*	1	98.81	586.00
43	AY544661	*Cheilymenia stercorea*	6	96.51	680.67
43	AY544654	*Aleuria aurantia*	2	94.92	744.00
43	DQ470966	*Eleutherascus lectardii*	1	98.67	528.00
44	EU011612	*Pichia finlandica*	9	93.14	563.56
45	AY789402	*Vibrissea truncorum*	5	96.71	730.80
45	DQ470957	*Loramyces macrosporus*	2	96.83	734.00
45	EF561637	*Polyozellus multiplex*	1	95.10	694.00
46	DQ667161	*Itersonilia perplexans*	2	94.43	583.50
46	DQ836005	*Udeniomyces puniceus*	1	94.20	293.00
47	DQ678074	*Davidiella tassiana*	7	99.96	745.43
48	AY548296	*Saitoella complicata*	8	91.78	605.63
49	EF550288	*Candida berthetii*	3	92.40	553.00
49	DQ986798	*Bacidina arnoldiana*	3	92.18	509.67
49	EF550329	*Pichia bimundalis*	3	90.89	578.00

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
50	AM259215	*Sistotrema coroniferum*	4	94.81	684.75
50	DQ915476	*Minimedusa polyspora*	2	97.05	760.50
50	AM259214	*Multiclavula vernalis*	2	96.24	732.00
51	AY646103	*Cryptococcus sp.CBS681.93*	7	98.56	734.00
52	DQ273799	*Lecophagus sp.ATCC56071*	6	96.89	713.00
52	DQ986799	*Umbilicaria aprina*	1	96.21	713.00
53	AY548296	*Saitoella complicata*	2	91.92	594.00
53	DQ986798	*Bacidina arnoldiana*	1	92.73	454.00
54	AY544650	*Geoglossum nigritum*	5	96.38	596.00
54	AY584641	*Xanthoparmelia conspersa*	2	96.07	521.00
55	DQ470967	*Pezicula carpinea*	4	96.96	515.75
55	AY789415	*Hyaloscypha daedaleae*	3	95.18	725.33
56	EU688966	*Mortierella indohii*	1	98.03	812.00
56	DQ273794	*Mortierella verticillata*	1	94.35	690.00
57	EF053601	*uncultured fungalcontaminant*	6	95.26	587.83
58	AY586646	*Clavaria fumosa*	6	94.26	749.50
59	DQ678082	*Alternaria alternata*	6	98.83	706.17
60	AY804191	*Chaenotheca chlorella*	3	93.27	708.00
60	AY640966	*Sarcogyne similis*	1	95.15	598.00
61	EF590327	*Fusarium oxysporum*	6	96.94	736.00
63	DQ273789	*Catenomyces sp.JEL342*	4	90.68	440.00
64	DQ518995	*Myxozyma udenii*	5	93.19	564.00
65	AY532967	*Porpidia sp.2-Buschbom14.9.2001*	4	91.64	699.50
65	AY532989	*Stephanocyclos henssenianus*	1	92.76	649.00
66	DQ470967	*Pezicula carpinea*	2	97.81	365.50
66	DQ470944	*Cudoniella clavus*	2	97.78	270.00
67	DQ518970	*Dipodascopsis anomala*	1	94.50	418.00
67	DQ518999	*Zygozyma smithiae*	1	94.43	377.00
67	DQ518987	*Myxozyma lipomycoides*	1	93.48	414.00
67	DQ273788	*Endogone lactiflua*	1	93.43	594.00
67	DQ518993	*Myxozyma nipponensis*	1	92.83	488.00
68	DQ273794	*Mortierella verticillata*	5	93.22	561.80

Table S2: Summary of best blast match within the main ITS1-MOTUs defined by MCL

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
1	DQ061918	*uncultured Russulaceae*	327	98.53	255.88
2	AJ534708	*Clavulinaceae sp. F46*	170	90.14	247.18
3	AJ557830	*Nectria mauritiicola*	134	99.97	224.92
4	DQ497970	*uncultured Atheliaceae*	100	90.60	180.68
5	DQ097873	*Hygrophorus albicastaneus*	92	94.04	296.21
6	DQ132834	*Papulaspora sp.*	85	120.96	232.53
7	EU597044	*uncultured ectomycorrhiza (Inocybe)*	80	94.14	231.00
8	EF126340	*fungal sp. WD32B*	71	99.94	217.00
9	EU240040	*Mortierella sp. WD10B*	69	99.28	204.78
10	EU030404	*uncultured fungus*	18	97.85	218.94
11	DQ273378	*uncultured Cortinariaceae*	64	91.44	324.97
12	EF434103	*uncultured fungus*	54	94.27	181.56
13	EU428773	*zygomycete sp. AM 2008a*	46	99.80	208.08
13	EU718661	*uncultured fungus*	1	100.00	208.00
14	EF434094	*uncultured fungus*	47	96.06	180.40
15	AY998789	*uncultured soil fungus*	44	85.30	185.48
17	EF521208	*uncultured fungus*	36	99.41	217.94
18	AF444377	*Cryptococcus tericola CBS 6435*	36	100.00	223.00
19	DQ093725	*Mortierella sp. Aurim 1236*	32	99.56	229.00
20	AY394919	*Cenococcum geophilum*	30	99.39	200.50
22	AY825525	*uncultured ectomycorrhiza(Thelephoraceae)*	24	96.54	263.96
25	AY825509	*uncultured ectomycorrhiza(Clavulinaceae)*	21	99.29	268.76
26	EF040834	*uncultured fungus*	20	98.87	220.90
27	AM902028	*uncultured basidiomycete*	7	99.81	224.00
27	AJ534713	*Cortinarius sp.P36*	7	98.66	224.00
27	DQ102683	*Cortinarius cf.saniosusHL90-235*	2	99.33	224.00
27	DQ102683	*Cortinarius cf.saniosusHL90 235*	1	99.55	224.00
27	DQ499461	*Cortinarius hinnuleoarmillatus*	1	99.11	224.00
27	AY669667	*Cortinarius helvolus*	1	96.96	230.00
28	EF093150	*Heliotale sp.*	19	98.29	219.11
28	EU645617	*uncultured ectomycorrhizal fungus*	19	97.38	219.11
29	EF601628	*Mortierella sp.SA1-3*	19	99.97	209.00
30	DQ421293	*uncultured soilfungus*	17	90.73	318.71
32	EU292660	*uncultured fungus*	16	92.37	183.44
34	EF218771	*uncultured Inocybe*	14	97.58	283.00
35	AJ878504	*Mortierella elongata*	14	94.77	213.00
36	EU877750	*fungal sp.60*	13	97.94	97.00
37	AJ279478	*Fusarium sp.5/97 45*	7	100.00	212.00
37	AJ279478	*Fusarium sp.5/97-45*	5	100.00	212.00
37	EU680533	*sordariomycete sp.7662*	1	100.00	212.00
38	AY781244	*ascomycete sp.olrim401*	9	89.91	228.00
38	AM260904	*uncultured fungus*	2	91.24	211.00
39	AY669664	*Cortinarius parvannulatus*	11	99.92	224.00
40	EU046038	*uncultured Filobasidiaceae*	11	99.52	229.00
41	EU826890	*uncultured soilfungus*	5	94.42	215.00
41	AY157495	*Mortierella cf.hyalinaTEA059*	5	93.64	210.40

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
42	EU292600	*uncultured fungus*	10	95.54	188.20
44	DQ469288	*Piloderma lanatum*	10	99.52	251.00
45	AJ534708	*Clavulinaceae sp.F46*	10	90.12	253.00
47	EF218817	*uncultured ectomycorrhiza (Sebacinaceae)*	10	99.87	233.00
49	DQ481983	*uncultured Atheliaceae*	9	91.51	178.00
50	EU202689	*uncultured Cortinariaceae*	5	98.20	299.40
50	EU819541	*Hebeloma crustuliniforme*	4	95.08	127.00
51	DQ420843	*uncultured soilfungus*	8	98.91	195.00
53	DQ421273	*uncultured soilfungus*	6	95.68	223.33
53	DQ182458	*uncultured Helotiales*	2	92.36	222.50
54	DQ102678	*Cortinarius saniosus*	8	99.93	219.50
55	DQ420853	*uncultured soilfungus*	7	99.82	241.00
55	AJ271629	*Mortierella alpina*	1	98.76	241.00
56	AJ581033	*uncultured basidiomyceteyeast*	4	95.15	227.00
56	EU046038	*uncultured Filobasidiaceae*	4	94.21	229.00
57	AF502621	*leaf litterascomycetestrainits029*	8	98.61	234.13
58	EF126325	*Mortierellales sp.GD18C*	7	94.95	235.00
59	AF440664	*Sebacina sp.MAS1*	8	93.39	227.00
60	EU819522	*Tomentella badia*	8	95.94	262.00
61	AF504842	*uncultured fungus*	8	99.76	212.00
62	DQ421142	*uncultured soilfungus*	8	91.63	271.75
65	EF031110	*Mortierella sp.W378*	7	99.33	234.57
66	EF601623	*Nectriaceae sp.PPn7-AFr*	6	99.74	195.00
66	EF601623	*Nectriaceae sp.PPn7 AFr*	1	100.00	195.00
67	TAU83487	*Thelephora americana*	7	98.17	254.86
68	EU819474	*Inocybe cf.soriora JMP0032*	6	82.57	327.00
69	EF040832	*uncultured fungus*	7	98.00	222.14
70	DQ420721	*uncultured soilfungus*	6	87.12	242.00
72	AM260897	*uncultured fungus*	6	97.69	216.00
75	EF521209	*uncultured fungus*	6	98.57	241.83
77	DQ317354	*Mortierellaceae sp.BC21*	6	99.05	228.00
79	EU523565	*Inocybe sp. PBM 2607*	6	99.31	288.17
80	AJ938166	*Geomyces pannorum var. asperulatus*	5	95.31	222.67
81	DQ420843	*uncultured soilfungus*	3	91.52	178.00
81	AY568066	*ascomycete sp.DAR73142*	3	90.62	199.00
84	TSU83470	*Thelephoraceae sp.'Taylor6'*	5	98.78	263.00
85	AM384928	*uncultured Archaeospora*	5	90.38	191.20
86	DQ421300	*uncultured soilfungus*	5	96.73	208.00
87	AM260863	*uncultured fungus*	5	87.21	204.80
88	AY970107	*uncultured basidiomycete*	5	98.57	210.00
89	AJ879659	*uncultured endophyticfungus*	4	97.26	210.00
89	DQ182427	*uncultured Helotiales*	1	98.64	221.00
91	AM260927	*uncultured fungus*	5	84.89	139.00
92	EU819474	*Inocybe cf.sorioraJMP0032*	5	93.60	125.00
93	AY656951	*uncultured mycorrhizalfungus*	4	90.08	262.00
93	DQ150127	*uncultured Thelephoraceae*	1	87.88	264.00
94	EU819474	*Inocybe cf.sorioraJMP0032*	5	88.49	318.20
95	EF434080	*uncultured fungus*	3	90.99	184.40

MOTUs	Genbank accession	Taxon	No of sequence	mean % identity	mean overlap
97	TSU83467	*Thelephoraceae sp.'Taylor2'*	5	99.54	263.00
98	AM902097	*uncultured basidiomycete*	5	97.91	320.00
99	AM902007	*uncultured basidiomycete*	5	85.71	301.00
100	EU428774	*zygomycete sp.AM-2008a*	5	97.72	202.00

III. Principaux résultats et discussion

1. Effet du régime d'enneigement

Les suivis par CE-SSCP des communautés bactériennes, fongiques, mais aussi crenarchaeotes (Fig. 14), montrent une structuration significative des communautés en fonction de leur statut thermique ou nival pendant la saison de végétation (Article C). Cette structuration est confirmée par la composition spécifique des communautés bactériennes et fongiques (Articles C et D, Annexe B). La disparité des communautés microbiennes de situation thermique ou nivale s'exprime par des différences d'abondance de ces groupes, plus ou moins favorisés par les conditions environnementales propres à chaque situation. Cependant, peu de groupes bactériens ou fongiques sont strictement spécifiques à l'une des deux situations, suggérant que les situations thermiques et nivales partagent un même pool d'espèces microbiennes (Article C, Annexe B).

Les communautés bactériennes du système thermique sont dominées par les phyla *Actinobacteria* et *Acidobacteria* (groupes SD3, SD6) alors que celles du système nival sont largement dominées par le groupe SD1 des *Acidobacteria* (Article C, Annexe B). De telles différences peuvent être dues à la qualité de la litière, plus récalcitrante en système thermique (Baptist, 2008) qui est susceptible de favoriser la présence d'Actinobactéries, connues pour dégrader le complexe lignocellulosique (Crawford, 1978). Mais c'est surtout le pH du sol, connu pour fortement structurer les communautés bactériennes (Fierer and Jackson, 2006; Fierer et al., 2007a), qui serait responsable ces différences. En effet, la croissance des Acidobactéries est favorisée dans des sols de pH plutôt acide (Lauber et al., 2008), comme ceux des sols des situations nivales (pH ~ 5,5). Plus particulièrement, la croissance de souches isolées du groupe SD1 est favorisée en milieux acides (Sait et al., 2006). En conséquence, le pH acide des sols de situation nivale semble agir comme un filtre sur la structure phylogénétique des communautés bactériennes (Annexe B).

De même, nous avons pu mettre en évidence la dominance de phylotypes de champignons ectomycorhiziens (*Cortinariaceae*, *Russulaceae*) et d'endophytes (*Pleosporales*) en situation thermique (Article D). L'abondance de tels groupes impliqués dans le transport et l'allocation des nutriments vers la végétation (Finlay, 2008; Green et al., 2008b) suggère que le milieu est peu fertile (Chapman et al., 2006). En revanche, les communautés fongiques de situation nivale sont beaucoup plus diversifiées, et composées de phylotypes appartenant à des groupes pour la plupart pauvrement documentés (*e.g.*

Atheliaceae, *Clavariaceae*) ou mal/non-identifiés (Article D). Cette diversité peut être due à une plus grande disponibilité des ressources en situation nivale (Waldrop et al., 2006). L'abondance des *Mortierellales* en situation nivale (Article D) corrobore cette hypothèse étant donné que cet ordre montre une forte dépendance aux substrats organiques dissous et facilement disponibles (Schmidt et al., 2008). **Ainsi, les systèmes thermiques et nivaux se distinguent non seulement par leur régime d'enneigement et leur végétation, mais aussi par la structure des communautés microbiennes du sol.**

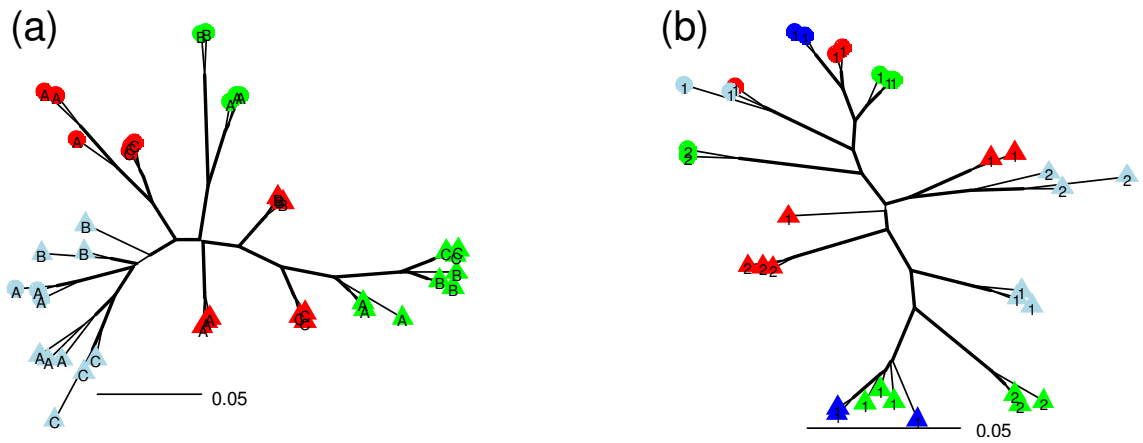

Figure 14 : Dendrogramme des variations saisonnières des communautés de crenarchaeotes en situations thermiques et nivales. (a) Suivi annuel sur les 3 sites A, B et C (indiqués dans les figurés). **(b)** Suivi biannuel sur le site B (année de suivi indiquée dans les figurés). Les situations thermiques sont représentées par des triangles, les situations nivales par des cercles. Les dates de prélèvements sont indiquées par des couleurs : bleu clair : Mai ; bleu : Juin ; vert : Aout ; rouge : Octobre. La distance d'Edwards et le Neighbor-Joining ont été utilisés pour construire les dendrogrammes. Les branches en gras sont significatives (valeurs de bootstrap > 500 sur 1000 retirages).

2. Dynamique saisonnière des communautés microbiennes de situations thermiques et nivales

Le suivi comparatif des communautés bactériennes, fongiques et crenarchaeotes en situations thermiques et nivales par CE-SSCP a révélé une augmentation de la similarité entre les communautés des deux situations en fin d'hiver (Article C, Fig. 14). Pour les communautés bactériennes et cette particularité s'explique principalement par la dominance du groupe SD1 (*Acidobacteria*) en situation thermique due à une acidification des sols à cette période (Annexe B). Pour les champignons, ce résultat est bien moins marqué, mais néanmoins confirmé par les données de séquençage de la région ITS1 (test de corrélation de

rangs de Kendall, $\tau = 0,212$; $P<0,05$). Cette similarité observée par CE-SSCP entre les communautés fongiques de situation thermique et nivale s'expliquent (i) par la présence de groupes peu abondants communs aux deux situations, et (ii) la différenciation de ces communautés par rapport aux communautés du reste de l'année (Article D). Les communautés crenarchaeotes et fongiques étant moins sensibles aux variations de pH (Nicol et al., 2004; Hogberg et al., 2007), il est possible que leur convergence dans les deux situations en hiver soit plutôt liée aux faibles températures, à l'absence d'activité du compartiment végétal. Ce phénomène peut également être imputé à un changement de la qualité/quantité des ressources après la dégradation de la matière organique durant la période hivernale. **Dans ces écosystèmes et indépendamment du statut thermique ou nival des communautés microbienne, l'hiver apparaît donc comme un événement fortement sélectif**.

Afin de déterminer le fonctionnement des systèmes thermiques en conditions hivernales, nous avons testé en conditions contrôlées l'impact du gel sur la cinétique de dégradation de tannins (extraits de *Dryas octopetala*) ajoutés à des carottes de sols provenant de ces systèmes (Annexe C). Nous avons ainsi pu mettre en évidence une absence du recyclage de l'azote organique imputé soit à une immobilisation de l'azote par les tanins *via* des phénomènes de complexation sous forme de complexe tannins-protéines, soit à une diminution des populations bactériennes impliquées dans le cycle de l'azote. En revanche, le recyclage du carbone organique s'est révélé plus efficace dans des conditions similaires aux conditions naturelles et est potentiellement dû à l'activité métabolique de champignons psychrophiles dominants dans ce type de conditions (Annexe C). Cette étude a également été menée sur les systèmes nivaux, pour lesquels les résultats ne seront pas abordés dans le présent document.

Le mois d'octobre apparaît également déterminant dans la structure phylogénétique des communautés bactériennes aussi bien dans les situations thermiques que nivales (Annexe B). Cet effet se traduit par le recrutement de *Streptosporangineae* chez les *Actinobacteria* et de *Pseudomonadales* et *Burkholderiales* chez les *Proteobacteria*. Chez les crenarchaeotes, l'effet de l'automne semble être le même que celui de l'hiver (Fig. 14) Certaines études ont mis en évidence l'effet prépondérant de la végétation et de la rhizosphère sur l'assemblage des communautés crenarchaeotes (Nicol et al., 2004; Sliwinski and Goodman, 2004). Il est donc possible que l'effet de l'hiver et l'automne sur les communautés crenarchaeotes résulte d'un arrêt de l'activité végétale. Ce résultat peut être imputé à une modification de la qualité/quantité des exsudats racinaires et/ou à une entrée massive de matière organique par

dépôt de litière, mais aussi aux lessivages automnales auxquels sont soumis ces systèmes. Chez les champignons, c'est le mois de juin en situation nivale qui montre la plus grande différence phylogénétique : la communauté est largement dominée par un phylotype dont l'identification n'est pas certaine (*Camarophyllus* ou *Hygrophorus*), rendant difficile l'interprétation d'un tel résultat (Article D).

Enfin, les communautés microbiennes des deux situations ne suivent pas la même dynamique d'une année à l'autre excepté durant les périodes clés c'est-à-dire l'hiver ; et l'automne chez les crenarchaeotes (Article C, Fig. 14b). Cette variation interannuelle, synchronisée par l'événement sélectif hivernal (ou automnal) peut résulter du recrutement de souches fonctionnellement similaires dans le pool d'espèces des deux situations (Article C). Ce phénomène montre que les communautés microbiennes alpines sont soumises à une forte perturbation, et que la résilience des communautés microbiennes suite à ces perturbations n'est pas cyclique. Il reste cependant à déterminer la similarité de la composition et de la fonction éventuelle des communautés microbiennes sur les deux ans de suivis afin d'évaluer l'impact écologique de ces patrons de résilience.

3. Considérations sur l'analyse de séquences

L'analyse des jeux de données de séquence des communautés fongique s'est révélée beaucoup plus difficile que celle des séquences bactériennes en raison de l'importante variabilité de la composition nucléotidique (ITS1 et gène de l'ARNr 28S) et de la taille (ITS1) des marqueurs moléculaires utilisés. Cette limitation nous a conduit à revisiter les méthodes d'analyses de séquence. Classiquement, l'analyse de jeux de donnés issu du clonage/séquençage repose sur l'alignement multiple des séquences. Or, les algorithmes d'alignement multiple sont des heuristiques (algorithmes approximatifs), et sont connus pour générer des erreurs dans l'alignement, notamment lors de l'insertion des « gaps » (Loytynoja and Goldman, 2008). Ce type de méthode ne peut donc pas être appliqué à des marqueurs moléculaires polymorphiques en taille comme l'ITS chez les champignons. De plus, ce biais peut avoir un impact significatif sur l'estimation de la diversité et l'établissement de la structure phylogénétique. Il est cependant rarement mentionné, ou contourné en corrigeant manuellement les alignements, pratiques qui, à l'heure du pyroséquençage, semblent tout à fait impensables.

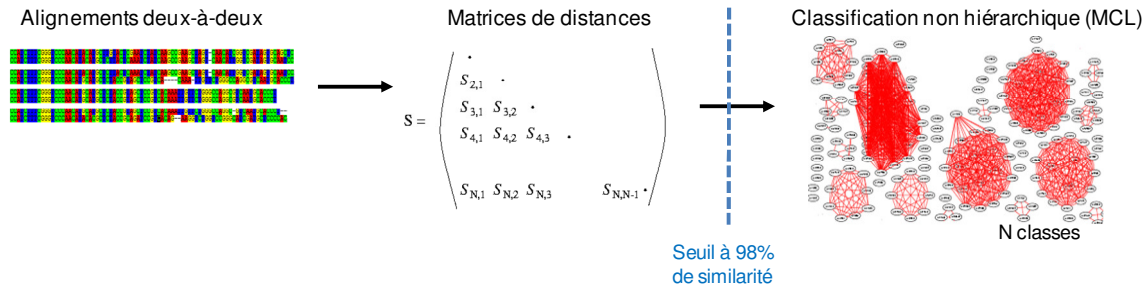

Figure 15 : Séquence d'analyse de séquences basée sur les alignements deux à deux et la classification non hiérarchique (Article D). MCL : Markov Clustering

La méthode d'analyse que nous proposons dans l'Article D (résumée dans la Fig. 15) présente l'avantage d'utiliser un algorithme d'alignement exact (Free End Gap) et de former des classes de séquences de façon robuste sans nécessiter l'établissement d'une phylogénie. Cette approche donne accès au même type de résultats que les approches classiques, à savoir la richesse, la diversité et la structure des communautés sans être soumise aux biais des alignements multiples. Elle peut également constituer une étape d'analyse préliminaire en vue d'une analyse plus profonde de la structure phylogénétique des communautés. Cette méthode est en cours d'optimisation et d'automatisation en vue de l'analyse de jeux de données issus du pyroséquençage (Eric Coissac, Christelle Melodelima et Guillaume Lentendu, LECA).

L'assemblage des communautés microbiennes est donc fortement influencé par le régime d'enneigement. Cet assemblage varie en fonction des saisons, notamment en hiver, et en automne chez les procaryotes. Durant ces périodes, les facteurs environnementaux tels que l'acidité du sol, la température, l'entrée de litière et le ralentissement de l'activité de la végétation filtrent l'assemblage des communautés microbiennes du sol. Ces résultats apportent des indices quant à l'impact que pourrait avoir un changement des régimes d'enneigement sur les communautés microbiennes et le fonctionnement des sols dans un contexte de réchauffement global.

Chapitre III – Vers une microbiologie du paysage : le cas des écosystèmes alpins

I. Problématique et démarche scientifique

1. Contexte général

La distribution des espèces dans l'espace géographique résultent **d'effets réciproques** des conditions environnementales (*e.g.* température, substrat, ressources), du compartiment biotique (*e.g.* composition en espèce, diversité), et des attributs biologiques de chaque composante de la communauté (*e.g.* préférences écologiques, traits fonctionnels, taux de dispersion). Les paysages que nous connaissons aujourd'hui sont donc façonnés par des mécanismes complexes s'opérant à différentes échelles spatiales et temporelles (cf. Introduction §III.2 et III.3, exemple de la distribution spatiale des espèces du sol Fig. 16) où la fragmentation du paysage joue un rôle déterminant (Ettema and Wardle, 2002). Alors que la biogéographie des macro-organismes a fait l'objet de nombreuses investigations, la distribution spatiale des communautés microbiennes demeure mal caractérisée, malgré leur implication dans les cycles biogéochimiques et leur action potentiellement positive ou négative sur les espèces environnantes, en particulier sur la végétation (cf. Introduction §I.3, (van der Heijden et al., 2008). Il est pourtant nécessaire de pouvoir prédire les patrons spatiaux des communautés microbiennes de façon à mieux évaluer l'impact des changements globaux sur le fonctionnement des écosystèmes.

Le manque de connaissances sur la biogéographie des micro-organismes a longtemps été lié à la difficulté de mettre en culture la majorité d'entre eux. Au début du XXème siècle, Beijerinck avait néanmoins pu observer que la croissance de la bactérie *Sarcina ventriculi,* une espèce ubiquiste, était fortement influencée par la nature chimique de son milieu (Beijerinck, 1911). Dans les années 1930, Baas-Becking avait pu également mettre en évidence l'influence de la concentration en calcium et en magnésium de l'eau de mer sur l'abondance d'une algue microscopique répandue dans l'eau de mer, *Dunaliella viridis* (Baas Becking, 1930). L'ensemble de ces observations a conduit Baas-Becking à

formuler l'hypothèse bien connue « *everything is everywhyere,* but*, the environment selects* » (Quispel, 1998). Beaucoup plus tard, les travaux de Finlay, basé sur l'observation microscopique de ciliés dans des échantillons provenant de diverses régions du monde, ont mis en évidence une distribution cosmopolite de ces organismes et ce, dans des proportions similaires (Finlay, 2002). Cependant, l'émergence des techniques basées sur la biologie moléculaire ont permis d'accéder aux organismes non cultivables, et l'idée de leur cosmopolitisme a été depuis largement remise en question. En effet, de nombreuses études tendent à montrer que les micro-organismes montrent des patrons spatiaux non aléatoires, et similaires dans une certaine mesure à ceux des macro-organismes (cf. Introduction §III.2, revue dans (Green and Bohannan, 2006). Particulièrement dans l'environnement extrêmement hétérogène que constitue le sol, la distribution spatiale des micro-organismes est susceptible d'être influencée à différents niveaux d'échelle spatiale et par la dynamique des interactions entre de nombreux facteurs biologiques et abiotiques (Fig. 16). L'enjeu actuel est donc de caractériser **quels sont les facteurs prédictifs des patrons spatiaux microbiens.**

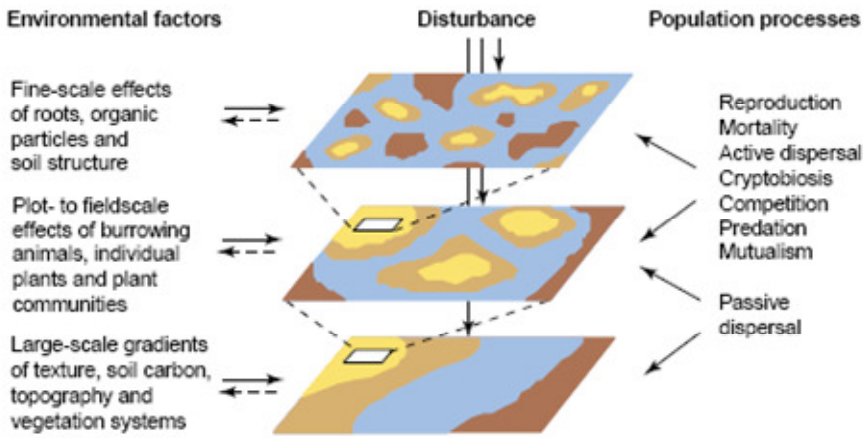

Figure 16 : Facteurs responsables de la distribution spatiale des organismes du sol. L'hétérogénéité spatiale des sols s'opère à différents niveaux d'échelle à travers une hiérarchie de facteurs environnementaux et de processus populationnels. Les perturbations environnementales jouent un rôle prépondérant dans cette distribution spatiale en modifiant les caractéristiques de l'habitat à petite ou grande échelle. La fragmentation du paysage résulte donc des effets réciproques des conditions environnementales et de la distribution spatiale des organismes du sol. *Source Ettema and Wardle (2002).*

La végétation constitue un facteur potentiellement prépondérant de la biogéographie des micro-organismes du sol. Par exemple, certains champignons associés aux racines, notamment les champignons ectomycorhiziens montrent une certaine spécificité à leur hôte et

à certaines conditions environnementales. La distribution géographique des espèces hôtes peut donc prédire celle des organismes associés à leurs racines (Allen et al., 1995; Berg and Smalla, 2009). De même, plusieurs études ont montré que la présence/absence, ou la densité du couvert végétal avait un impact significatif sur la structure des communautés bactériennes (Kowalchuk et al., 2002; Yergeau et al., 2007). En effet, la végétation constitue l'apport principal des ressources dans le sol par dépôt de litière et exsudation racinaire. Or, la qualité et la quantité de ces apports sont variables d'une espèce végétale à l'autre, modifiant ainsi les propriétés physico-chimiques du sol (Eviner and Chapin, 2003), et peuvent donc influencer l'assemblage des communautés microbiennes. Ainsi, différentes pratiques des terres, traduites par un changement de la composition floristique, ont un impact sur les communautés bactériennes et fongiques car elles montrent des caractéristiques chimiques du sol différentes, telles que le pH ou la teneur en phosphore (Kasel et al., 2008; Lauber et al., 2008). Aussi, le précèdent chapitre a montré un effet significatif du couvert végétal sur l'assemblage des communautés microbiennes, malgré l'échelle locale de l'étude. Bien que ces informations soient primordiales dans la compréhension des patrons spatiaux microbiens, l'impact de la couverture végétale demeure mal établi.

La flore alpine est principalement constituée d'arbrisseaux nains, de graminoïdes (principalement des *Cyperaceae* et des *Poaceae*) et d'espèces herbacées, la plupart étant des dicotylédones à rosettes ou en coussinets (Körner, 1995). Les **gradients mésotopographiques** (Fig. 17 a et b) présents dans les écosystèmes alpins conduisent à une distribution hétérogène du manteau neigeux en hiver. Déterminant la durée de la saison de végétation, l'hétérogénéité des régimes d'enneigement est responsable de changements dramatiques de la flore sur de très courtes distances. Ces cortèges floristiques ont été décrits comme ayant des caractéristiques écophysiologiques variant de manière consistante le long du gradient d'enneigement (Choler, 2005). L'amplitude des gradients environnementaux dans le paysage alpin en fait donc un modèle de choix pour caractériser les patrons spatiaux des communautés microbiennes à l'échelle du paysage, ainsi que les facteurs responsables d'une telle distribution.

2. Objectifs de l'étude

Notre étude s'est concentrée dans un bassin versant de haute altitude (1900 à 2800 m) et s'étend de l'étage subalpin à l'étage alpin. Le substrat du site d'étude est uniformément constitué de flyshs (schistes argileux calcaires). Nous nous sommes concentrés sur douze

habitats répartis sur l'ensemble du bassin versant (Fig. 17 c, Table 2). Ces habitats sont caractérisés par des communautés végétales différentes qui se répartissent le long du gradient d'enneigement et, en conséquence, embrassent l'ensemble de la flore alpine.

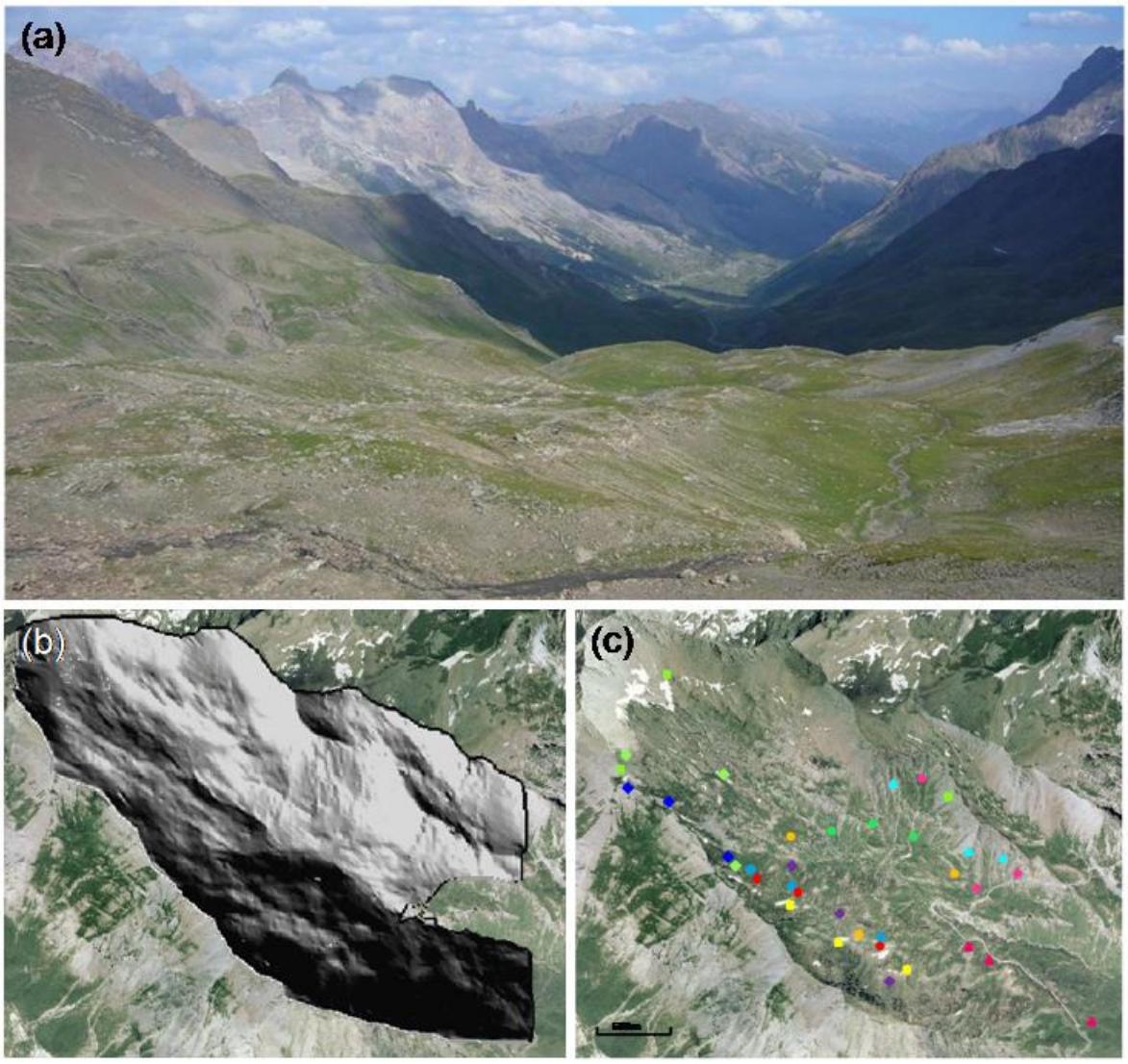

Figure 17 : Site de l'étude (Vallon de Roche Noire, commune de Le Monêtier les Bains). (a) Vue d'ensemble du bassin versant *Photographie : D. Lejon.* **(b)** Pente et exposition du site *Source* : *P. Choler*. **(c)** Points d'échantillonnages. Les correspondances des symboles et des couleurs sont indiquées dans la Table 1.

Notre objectif a été de déterminer (i) si les patrons spatiaux des communautés microbiennes suivaient des patrons spatiaux similaires à la végétation, et (ii) quelles étaient les facteurs expliquant au mieux cette variabilité spatiale à l'échelle du paysage. Dans ce but, la campagne d'échantillonnage s'est faite sur deux jours, au milieu du mois d'Août, pendant le pic de la saison de végétation dans cette région. Nous avons caractérisé la

composition floristique de chaque point d'échantillonnage et leurs attributs (diversité, biomasse aérienne). Nous avons également déterminé les propriétés chimiques des sols (pH et teneur en matière organique) et les caractéristiques topographiques et climatiques à partir de modèles numériques. Les communautés microbiennes ont été suivies par CE-SSCP. Les résultats obtenus sur certains points d'échantillonnage (EN) ne seront pas présentés dans le présent chapitre en raison de l'indisponibilité des données floristiques.

Table 2 : Communautés végétales étudiées. [1]plantes pouvant vivre dans la neige. [2]plantes poussant à des températures modérées. [3]plantes adaptées au froid. Les symboles correspondent à ceux des points d'échantillonnage indiqués dans la Fig. 17

Symboles	Type de communauté	Espèces dominantes
●	Pelouse alpine chionophile[1]	*Carex foetida, Alchemilla pentaphyllea, Salix herbacea*
●	Pelouse subalpine/alpine	*Carex sempervirens, Trifolium alpinum*
■	Eboulis à manteau neigeux persistant	*Ranunculus glacialis*
■	Eboulis sur pente exposée Sud	*Crepis pygmeae, Doronicum grandiflorum*
♦	Pelouse subalpine/alpine mesophile[2]	*Festuca violacea, Alchemilla filicaulis, Geum montanum*
●	Pelouse subalpine mesophile[2]	*Festuca paniculata*
▲	Pelouse écorchée subalpine sur éboulis	*Helictotrichon sedenense, Festuca violacea*
♦	Pelouse alpine thermique sur crête	*Kobresia myosuroides, Dryas octopetala*
♦	Pelouse alpine thermique	*Kobresia myosuroides, Sesleria coerulea, Carex rosae*
♦	Lande psychrophile	*Salix retusa, Salix reticulata*
●	Pelouse subalpine	*Trifolium pratense, Geranium sylvaticum*
▲	Lande subalpine	*Vaccinium uliginosum, Vaccinium_myrtillus*

II. Contribution scientifique

Article E: Zinger L., Bouasaria A., Baptist F., Geremia R.A., Choler P.: Landscape-scale distribution of microbial communities in alpine tundra in relation to plant cover and environmental conditions. *in preparation.*

Chapitre III – Article E

Landscape-scale distribution of microbial communities in alpine tundra in relation to plant cover and environmental conditions

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In preparation

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Key words: fungi; bacteria; crenarchaeotes; microbial biogeography; soil pH; CE-SSCP; Single Strand Conformation Polymorphism.

Running title**:** Biogeography of alpine soil's microbial communities

ABSTRACT

The biogeography of micro-organism is still debated, despite their importance in ecosystem process and the needs to predict their spatial distribution in a global change context. Here, we report a landscape-scale survey of the spatial distribution of soil crenarchaeal, bacterial and fungal communities in alpine tundra. We attempted to assess the similarity degree between spatial patterns of microbial and plant communities and to characterize the abiotic factors involved in their spatial distribution. The soil microbial communities were investigated by using CE-SSCP (Capillary Electrophoresis Single Strand Conformation Polymorphism) in eleven plant communities representative of the alpine flora. We found that microbial communities and plant cover spatial patterns were correlated and organized along a soil pH gradient. While the soil pH was strongly determinant in the biogeography of bacterial, and in a certain extent, of crenarchaeal communities, fungal communities were found correlated with soil pH, above-ground live phytomass and slightly with soil organic matter, suggesting that their spatial distribution results from complexes mechanisms in which soil pH and soil fertility are involved. This study also highlighted the lack of relation between microbial communities' similarity and geographic distance. Hence, at the landscape scale, microbial communities exhibit spatial variations linked to environmental conditions (here, soil properties and plant cover), rather than to geographic distance, supporting the hypothesis of Baas-Becking at moderate spatial scales.

1. INTRODUCTION

Micro-organisms play a central role in biogeochemical processes, especially in the carbon cycle. Understanding and predicting the spatial distribution patterns of soil microbial communities at the landscape scale is thus one of the key issues in a global change context, and will provide a more comprehensive overview of ecosystem processes. Given that the majority of micro-organisms are still uncultured, their spatial distribution has long been poorly documented compared to macro-organisms. The first idea of a microbial biogeography was proposed by Baas-Becking and Beijerinck, whom assumed that "everything is everywhere, but, the environment selects". A more recent statement argued that micro-organisms are cosmopolitan due to their huge abundance and their potential high dispersal rate (Finlay, 2002). However, with the recent development of molecular technologies, an increasing body of literature has highlighted the strong variability of microbial community at various spatial scales (review in Green and Bohannan, 2006; Hughes Martiny et al., 2006). At

global scales, several surveys demonstrated the speciation of some microbial strains (Cho and Tiedje, 2000; Taylor et al., 2006), and at the province scale (*sensu* Hughes Martiny et al., 2006), numerous studies provided evidences of the influence of habitat characteristics on microbial communities, such as soil pH, resource availability or land use type (Fierer and Jackson, 2006; Waldrop et al., 2006; Kasel et al., 2008; Lauber et al., 2008).

Plant cover has also likely a role in the microbial communities' spatial distribution. First of all, the absence or presence of roots, as well as the plant species, strongly effects on the microbial communities' structure (Kowalchuk et al., 2002; Yergeau et al., 2007). Indeed, the rhizosphere carbon flow provides high amounts of readily used organic substrates for microbes, but can also contains signal molecules mimicking those of micro-organisms whereby they regulate the population density and gene expression (review in (Standing et al., 2005). Plant species also display a large variety of traits influencing soil organic matter quality and quantity though litter or root exudates (Eviner and Chapin, 2003). This feature has likely effects on the soil food web and soil properties (review in Wardle et al., 2004; Berg and Smalla, 2009). In return, soil microbes can have an impact on both plant productivity and floristic composition either by modifying nutrient availability for plant, though organic matter mineralization or N_2 fixation, or by promoting/excluding plant species through mutual/symbiotic or pathogenic associations (review in van der Heijden et al., 2008). However, despite the key role of plant cover in the below-ground system functioning, only a few surveys attempted to characterize the effects of the above-ground floristic composition on microbial community assembly *in situ* (but see Mummey and Stahl, 2003; Zak and Kling, 2006; Wallenstein et al., 2007; Bjork et al., 2008).

In alpine tundra, landscapes are strongly marked by mesotopographical gradients controlling the snow cover duration, which triggers strong habitats heterogeneity, ranging from long lasting snowbeds to snow-free crests. As snow cover regime determines the growing season length, alpine tundra exhibits a high plant species turnover at reduced scales (review in Körner, 1995), of which traits have been reported to consistently vary along the mesotopographical gradient (Choler, 2005). Consequently, alpine landscapes also display heterogeneous soil properties and biogeochemical processes (Olear and Seastedt, 1994; Litaor et al., 2001) and thus constitute a suitable model to assess the landscape-scale distribution of microbial communities in relation to plant cover and environmental conditions. An earlier paper reported that snow cover regimes and vegetation had minor influences on the spatial distribution of microbial PLFA (Phospholipid Fatty Acid) profiles (Bjork et al., 2008). At the opposite, (Zak and Kling, 2006) highlighted the effect of three plant cover types on microbial

PLFA profiles in arctic tundra, and our previous study revealed strong contrasts in CE-SSCP (Capillary Electrophoresis Single Strand Conformation Polymorphism) microbial profiles obtained from two plant communities located at the both extreme of the mesotopographical gradient (Zinger et al., in press).

Here, we attempted to determine the similarity degree of the landscape-scale distributions of fungal, bacterial and crenarchaeal communities and plant communities' ones. The study was carried out along the mesotopographical gradient in a small watershed located in French Alps. We followed-up eleven plant communities, encompassing most of the floristic gradient of the alpine tundra, in order to estimate the magnitude of the spatial co-variation of both plant and microbial communities. Soil microbial communities were characterized by using CE-SSCP method on ribosomal RNA genes. We also examined the contribution of climatic, topographic, soil chemistry and some plant communities' attributes in the microbial biogeography.

2. MATERIAL AND METHODS

Soil sampling: The study area is located in the Grand Galibier Massif in the French South-Western Alps (Lieu-dit Vallon de Roche Noire, commune de Le-Monêtier-les-bains, France; 45°0.05'N, 06°0.38'E). It corresponds to a high-elevation watershed, ranging from 1900m to 2800m, with the main slopes oriented to south-west and north-east (Fig. 1). The area is located above the treeline and extends from the subalpine to the alpine belt. The vegetation is composed of a mosaic of herbaceous and heath communities. Based on previous phytoecological studies (Choler and Michalet, 2002), eleven plant communities that cover most of the vegetated area has been selected (Table 1). These plant communities are easily distinguished by two or three dominant vascular species, usually grasses, sedges or shrubs (Table 1). Based on this analysis, three sampling units (only two for ES) per plant community separated by at least 100 m were chosen. The 32 sampling units consisted in plots of 5×5 m^2 and their location and description are indicated in Table 1 and Fig. 1. Soil collection was carried out on at the peak of standing phytomass in July 2007. In each sampling unit, three soil spatial replicates were collected from the top 10 cm of soil in each sampling unit. All soils samples were then homogenized, sieved at 2 mm, and kept at -20°C for subsequent analyses.

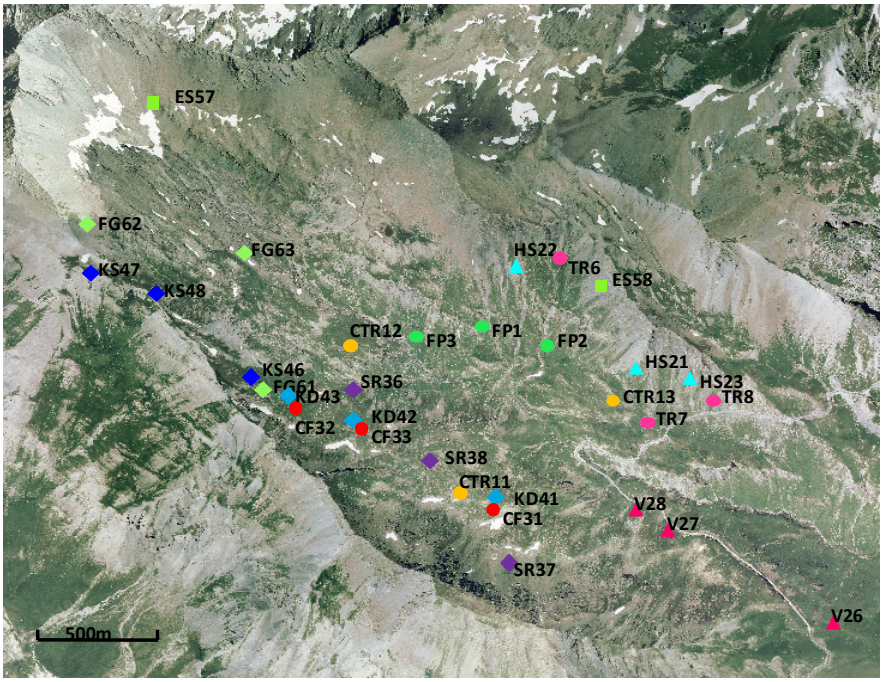

Figure 1 : Satellite photography of the Vallon de Roche Noire (massif du Galibier, France) and sampling units location. See Table 1 for symbols and code correspondences.

Site characterization: The floristic composition of each sampling unit was assessed by visually estimating the percentage cover of vascular plant species. We used the following scale: 1: <5%; 2: 5-10%; 3: 10-25%; 4: 25-50%; 5: 50-75%; : >75%. Based on this scale, we obtained a floristic table containing the occurrence frequency of 191 species in each sampling unit. The evenness of plant communities was assessed from this table by using the converse of Simpson index as suggested by (Smith and Wilson, 1996). Standing crop phytomass was collected in squares plots of 20 × 20 cm except in FP, where square plots of 50 × 50 cm were used. Live and dead material were separated, incubated at 85°C for 48h, and weighted for phytomass measurements. The topological and variables were extracted from a digital elevation model at 10 × 10 m of resolution. Climatic variables were extracted from the French meteorological model Aurelhy (Météo-France, (Benichou and Le Breton, 1987) based on interpolation of measurements at 100 × 100 m of resolution. Soil pH was measured after mixing 5 grams of soil with 12.5 ml of distilled water (adapted from Yan et al., 1996). Soil organic matter content (SOM) was determined by loss-on-ignition according to Schulte and Hopkins (1996).

Table 1: Description of the eleven plant communities studied. Evenness was calculated as the converse of Simpson index, and was not included in multivariate analyses. Evenness values represent means (SD) of plant community triplicates.

Plant community designation	Sampling units	Plant community description	Dominant species	Evenness
● CF	31, 32, 33	Chionophilous alpine meadow	*Carex foetida, Alchemilla pentaphyllea, Salix herbacea*	0.31 (0.12)
● CTR	11, 12, 13	Subalpine/alpine meadow	*Carex sempervirens, Trifolium alpinum*	0.24 (0.14)
■ ES	56, 57, 58	Scree community on southern exposed slopes	*Crepis pygmeae, Doronicum grandiflorum*	1
♦ FG	61, 62, 63	Mesophilous subalpine/alpine grassland	*Festuca violacea, Alchemilla filicaulis, Geum montanum*	0.24 (0.06)
● FP	1, 2, 3	Mesophilous subalpine grassland	*Festuca paniculata*	0.27 (0.10)
▲ HS	21, 22, 23	Open subalpine meadow on screes	*Helictotrichon sedenense, Festuca violacea*	0.31 (0.01)
♦ KD	41, 42, 43	Fellfield	*Kobresia myosuroides, Dryas octopetala*	0.22 (0.05)
♦ KS	46, 47, 48	Thermic alpine meadow	*Kobresia myosuroides, Sesleria coerulea, Carex rosae*	0.26 (0.09)
♦ SR	36, 37, 38	Psychrophilic dwarf willows community	*Salix retusa, Salix reticulata*	0.14 (0.01)
● TR	6, 7, 8	Subalpine tall herb community	*Trifolium pratense, Geranium sylvaticum*	0.26 (0.06)
▲ V	26, 27, 28	Subalpine heath	*Vaccinium uliginosum, Vaccinium_myrtillus*	0.18 (0.07)

Microbial community analyses: Soil DNA extractions were carried out in triplicates from 0.250 g of each soil sample (wet mass) with the PowerSoil-htp^{TM} 96 Well Soil DNA Isolation Kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) according to the manufacturer instructions. DNA concentration was quantified using the NanoDrop ND-1000 (NanoDrop technologies). DNA extracts of spatial replicates were pooled in order to limit the effects of soil spatial heterogeneity (Schwarzenbach et al., 2007). Bacterial 16S rRNA genes were amplified with the primers W49 and W104-FAM labeled (Delbes et al., 1998). Crenarcheaotal communities were assessed using primers targeting 16S rRNA gene, namely 133FN6F-NED labeled and 248R5P (Sliwinski and Goodman, 2004). Fungal ITS1 was amplified with the primers ITS5 and ITS2-HEX labeled (White et al., 1990).

The PCR reactions (25 µl) consisted in 2.5 mM of MgCl2, 1U of AmpliTaq GoldTM buffer, 20 g l-1 of bovine serum albumin, 0.1 mM of each dNTP, 0.26 mM of each primer, 2 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Courtaboeuf, France) and 10 ng of DNA template. The PCR reaction was carried out as follows: an initial step at 95°C (10 min), followed by 30 cycles at 95°C (30 s), 56°C (15 s) and 72°C (15 s), and final step at 72°C (7 min). PCR products were checked on a 1.5% agarose gel, and amplicons of each microbial community from the same sampling unit were pooled to perform multiplex CE-SSCP. Capillary electrophoresis-SSCP conditions were performed on an ABI Prism 3130 XL genetic analyzer (Applied Biosystems, Courtaboeuf, France), as previously described in (Zinger et al., 2008). The obtained CE-SSCP profiles were normalized in order to reduce the variations of fluorescence intensity between profiles.

Statistical analyses: All statistical analyses were carried out with the R software (R_Development_Core_Team, 2007). We first examined the differences of environmental conditions between sampling units were assessed by performing a principal component analysis (PCA) with 7 variables, namely elevation, slope, orientation to south, annual radiations, soil pH, % SOM and live phytomass. For this purpose, live phytomass values were log-transformed and all variables were normalized. Distances between sampling units' environmental conditions were calculated by using canonical distance. The geographic distances (straight-line distance between sampling units) between sampling units were assessed from Lambert converted GPS coordinates by using Euclidean distance. Similarities between sampling units in their floristic composition were calculated using the Edwards' distance. CE-SSCP microbial profiles were log-transformed, and their similarity was assessed by using canonical distance.

To determine if microbial communities were characteristic to the above plant community, we calculated the standardized effect of mean nearest neighbor distances (MNND, Webb et al., 2002). Although this method is usually used for phylogenetic studies, we used the standardized effect of MNND to test if the mean distance between microbial communities from a same plant community type is smaller than in a null model. This null model corresponds to the mean of MNND obtained by randomizing 1000 times their plant community belonging. This analysis returns a negative value if microbial communities are similar within the same condition. We used nonmetric multidimensional scaling (NMDS) for the ordination of sampling units for plant, crenarchaeal, bacterial, and fungal communities. We then performed Mantel tests to determine if geographic distances or environmental variables were associated with the patterns of similarity between plant, bacterial, fungal, or crenarchaeal communities. We also examined the co-variations of similarity patterns between plant, bacterial, fungal, and crenarchaeal communities. Mantel test were conducted by using the Spearman rank correlation method with 1000 permutations.

3. RESULTS

Environmental characteristics of sampling units

The characteristics of plant communities and location of sampling units are given in Table 1, Fig. 1. The Fig. 2a displays the Non-metric Multidimensional Scaling (MNDS) of floristic composition of sampling units. Fig 2b corresponds to the principal component analysis (PCA) of the 7 environmental variables measured in this study, and explained 60% of the variance. The PCA analysis showed the similarity of environmental conditions within a same plant community except for TR and FG, the last one being also variable in its floristic composition (Fig. 2).

Along the PCA axis 1, plant communities are separated by elevation, slope and SOM, which were not correlated (Pearson's $r = -1.74$ to 0.73, $P > 0.09$), and soil pH, which displayed strong correlation with the aforementioned variables (Pearson's $r = 2.43$, 2.40 and -3.75 respectively, $P < 0.02$). The elevation of plant communities ranged from 2227 m to 2818 m, with FG, ES and KS displaying the highest elevations whereas V displayed the lowest one. This elevation gradient is strongly correlated with a temperature gradient (data not shown). SOM content was the lowest in ES soils (3.4%), the highest in CTR and V (~29%) and correlated with the floristic composition (Table 3). Finally, the slope, ranging from 8.05° to 41.5°, was found variable for a same plant community type, but appeared much higher in FG,

HS, SR, FP, TR and ES and slightly co-varied with the floristic assemblage (Table 3). The studied site also displays a strong gradient of soil pH, ranging from 4.9 in CTR to 7.9 in ES, which co-varied with the floristic composition of plant communities (Table 3).

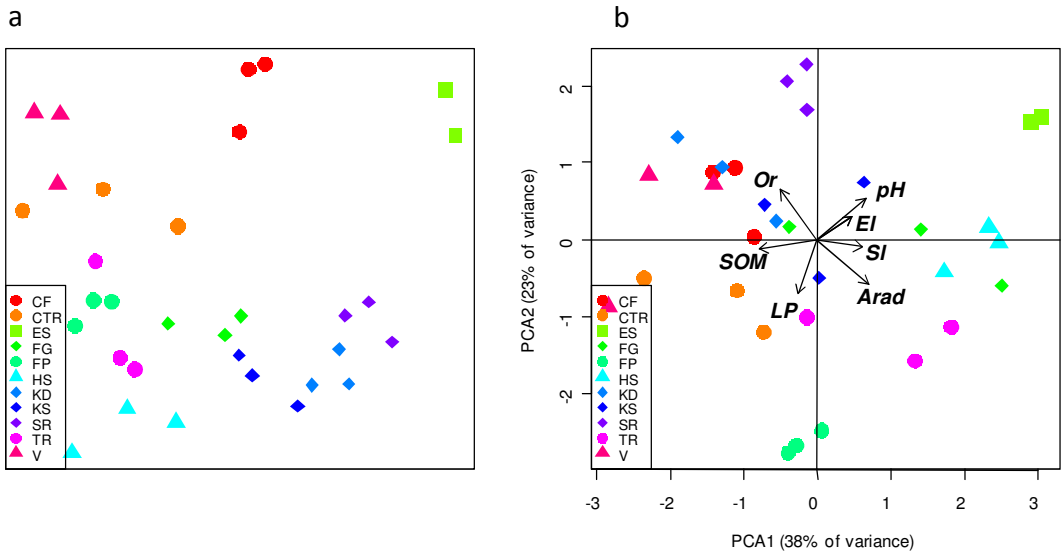

Figure 2: **(a)** Nonmetric multidimensional scaling (NMDS) ordination of the floristic composition in sampling units. The stress value of NMDS ordination is 0.21 **(b)** Principal Component Analysis (PCA) of the 7 measured environmental variables in the 32 sampling units. Legend indicates the plant communities described in Table 1. Vectors indicate environmental variables (eigenvector scores > 0.4): El: elevation; Sl: slope; Or: orientation to south; Arad: annual radiations; pH: soil pH; SOM: % soil organic matter; LP: live phytomass. See Table 1 for symbols and code correspondences.

The PCA axis 2 revealed mostly a gradient of live phytomass, limited in HS and ES (at most 143 $g.m^{-2}$), which display scattered plant cover. In contrast, live phytomass was maximal in FP (2293 $g.m^{-2}$). This variable was correlated with annual radiations (Pearson's r = 2.34, P = 0.025), which divided the plant communities in two main groups: SR, EN, V, CF and KD displayed annual radiations ranging from 11223 to 18236 $kJ.m^2.d^{-1}$ whereas HS, TR, FP, ES, FG, KS and CTR displayed higher annual radiations ranging from 16426 to 23038.$kJ.m^2.d^{-1}$. Orientation and annual radiations were also found significantly correlated (Pearson's r = -4.32, P < 0.001) and co-varied with the species composition of plant communities (Table 3).

As expected, Mantel tests showed high correlation between elevation and geographic distances (Spearman's ρ = 0.74, P < 0.001), and weaker correlations of orientation, annual radiations or % SOM with geographic distances (Spearman's ρ = 0.11 to 0.18, P < 0.05).

Others environmental variables were not found correlated with geographic distance (Spearman's ρ = -0.04 to 0.04, $P > 0.2$). Consequently, floristic composition of plant communities was also found slightly correlated with geographic distances (Table 3).

Microbial communities and plant cover

The nonmetric multidimensional scaling (NMDS) ordinations of fungal, bacterial and crenarchaeal communities are shown in Fig. 3. The standardized effect of mean nearest neighbor distances (MNND, Webb et al., 2002) was used to assess the analogy of microbial communities from a same plant community type (Table 2). Most of values were found negative, suggesting a tendency of microbial communities to be similar in one plant community, but were seldom found significant. Crenarchaeal communities were no or rarely amplified in V, CTR, FP and CF, and displayed a strong dispersed structure (Fig. 3). The standardized effect of MNND revealed, however, the similarity of crenarchaeal communities in ES, and SR. The NMDS ordination of bacterial communities was almost linear (Fig. 3), and bacterial communities were found similar in FG and TR (Table 2). Fungal communities of plant community triplicates were found quite dispersed (Fig. 3) but were significantly similar in CF, CTR, KS, and V (Table 2).

Table 2: Standardized effect size of mean nearest neighbor distance (MNND) in microbial communities according to plant community type. Significance of the standardized effect size of MNND vs. null model was obtained with 1000 permutations and is indicated by stars: * P<0.05, ** P<0.01, *** P<0.001. This test indicates if microbial communities are clustered by plant community type. N.d.: not defined.

Plant community	Crenarchaeotes	Bacteria	Fungi
CF	N.d.	-1.04	-1.81 *
CTR	N.d.	-0.24	-2.15 *
ES	-2.32 *	-1.08	-1.26
FG	0.29	-1.61 **	-1.09
FP	N.d.	-1.23	-0.24
HS	-1.38	-0.71	1.34
KD	-0.60	-1.24	-0.63
KS	-0.10	-0.81	-2.76 **
SR	-2.02 *	0.57	-1.46
TR	-0.63	-1.53 **	-0.94
V	N.d.	-1.13	-3.11 **

Although the microbial communities seemed poorly characteristic to the plant community type, the Mantel tests confronting fungal, bacterial or crenarchaeal communities's composition against the floristic composition of sampling units was found significant (Table

3). In the same way, the evenness of plant communities was found slightly correlated with microbial communities (Table 3). Additionally, bacterial and crenarchaeal communities were found correlated (Spearman's ρ = 0.38; P = 0.01). Interestingly, none of microbial communities were found related to geographic distances (Table 3).

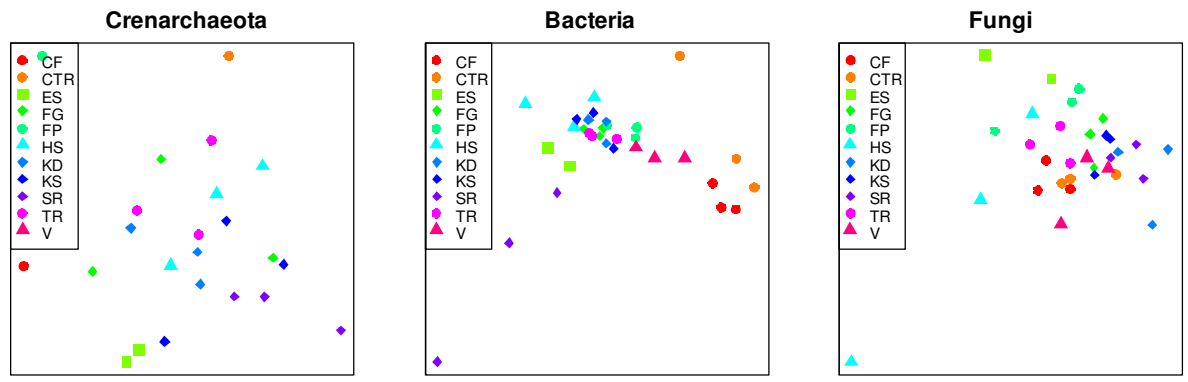

Figure 3: NMDS ordination of crenarchaeal, bacterial and fungal communities (assessed by CE-SSCP [Capillary Electrophoresis Single-Strand Conformation Polymorphism]). The stress values of NMDS ordination are 0.14, 0.06 and 0.18 for crenarchaeal, bacterial and fungal communities respectively. See Table 1 for symbols and code correspondences.

Microbial communities and environmental conditions

The Mantel test was used to determine which of the environmental measures co-varied with microbial communities' composition (Table 3). Despite the apparent dispersed ordination of crenarchaeal communities, they were slightly correlated with soil pH (Table 3, Fig. 4). Bacterial communities were poorly correlated with annual radiations; but displayed a strong correlation with soil pH, which is responsible of the linear distribution of bacterial communities in the NMDS ordination (Fig. 3, 4). Finally, fungal communities displayed faint correlations with annual radiation and SOM and were most correlated with soil pH and live phytomass (Table 3). While the effect of live phytomass was poorly visible in the NMDS ordination, the soil pH appeared correlated with the axis 2 of NMDS ordination (Fig. 4).

Table 3: Correlations between crenarchaeal, bacterial and fungal communities with environmental variables, floristic composition and geographic distances. Environmental variables were normalized. Correlation significances were assessed with 1000 permutations and are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001. N.d.: not defined.

	Spearman rank ρ values			
Variables	Plant	Crenarchaeotes	Bacteria	Fungi
Environmental conditions				
Single variable				
Elevation	0.04	0.02	-0.18	-0.001
Slope	0.12 *	-0.09	0.10	-0.04
Orientation	0.20 **	0.13	0.11	0.02
Annual radiations	0.23 ***	0.02	0.16 **	0.14 **
Soil pH	0.40 ***	0.35 ***	0.62 ***	0.28 **
% SOM	0.36 ***	0.09	0.06	0.15 *
Live phytomass	0.04	0.12	-0.08	0.32 **
Multiple variables	Soil pH + %SOM		soil pH + annual radiations	live phytomass + soil pH
	0.52 ***		0.54 ***	0.39 ***
	Soil pH + %SOM + annual radiations			live phytomass + soil pH + % SOM
	0.58 ***			0.36 ***
Floristic composition		0.43 ***	0.44 ***	0.30 **
Evenness		0.29 *	0.18 *	0.19 *
Geographic distance	0.14 *	-0.07	-0.09	0.11

4. DISCUSSION

Increasing evidences support the fact that microbial communities exhibit strong spatial and temporal variability (review in Bardgett et al., 2005; Hughes Martiny et al., 2006), but the drivers responsible of such patterns are still poorly characterized. In this context, the fragmented landscapes of alpine tundra, in terms of floristic composition, climatic and soil conditions as well as ecosystem processes (Olear and Seastedt, 1994; Körner, 1995; Litaor et al., 2001; Choler, 2005), offers good opportunities to assess the causes responsible of the spatial distribution of micro-organisms.

The eleven plant communities surveyed here encompass most of the floristic diversity of alpine tundra, and are distributed along strong environmental gradients (Fig. 2, Table 3). Clearly, the spatial distribution of plant communities studied here is partly caused by sunshine, but also both act on/result from the spatial heterogeneity of soil pH and soil organic matter (Table 3). There are two main reasons of such a feature. First, in our study site, the bedrock is calcareous shales and consequently basic. Young soils, displaying scattered plant cover (*i.e.* ES, HS, SR), are quite pH-neutral and relatively poor in SOM. In contrast mature soils, covered by dense plant cover, are the most acidic, due to a smaller effect of bedrock, and display generally higher SOM content (*i.e.* CF, CTR, V, FP). Soil pH has been previously reported to co-vary with soil C and N dynamics (Kemmitt et al., 2006). In the same way, (Gough et al., 2000) argued that the co-variation of soil pH and floristic composition resulted from complex mechanisms that influenced nutrient availability. Also, (Fierer et al., 2007) observed a correlation between pH and dissolved organic carbon in fine benthic organic matter and (Bardgett et al., 1999) highlighted a decrease of soil pH with the addition of N. Secondly, plant communities are dominated by species differing in their eco-physiological traits, which can affect soil properties according to carbon compounds contained in their litter or roots exudates (Eviner and Chapin, 2003; Bais et al., 2006). Hence, all these biotic and abiotic factors are intimately linked, and as soil micro-organisms are strongly involved in the plant-soil feedbacks (review in (Wardle et al., 2004; van der Heijden et al., 2008), they would exhibit coherent spatial patterns to those of plant communities and soil properties.

The most striking result we have obtained is that soil pH explains in a certain extent the spatial distribution of all microbial groups studied here (Fig.4, Table 3). Soil pH has already been described as a good predictor of bacterial biogeography in soils and fine benthic organic matter (Fierer and Jackson, 2006; Fierer et al., 2007; Lauber et al., 2008). Our results confirm

this trend in alpine tundra soils for both prokaryotes and fungi, but above all for bacterial (Fig. 3, 4).

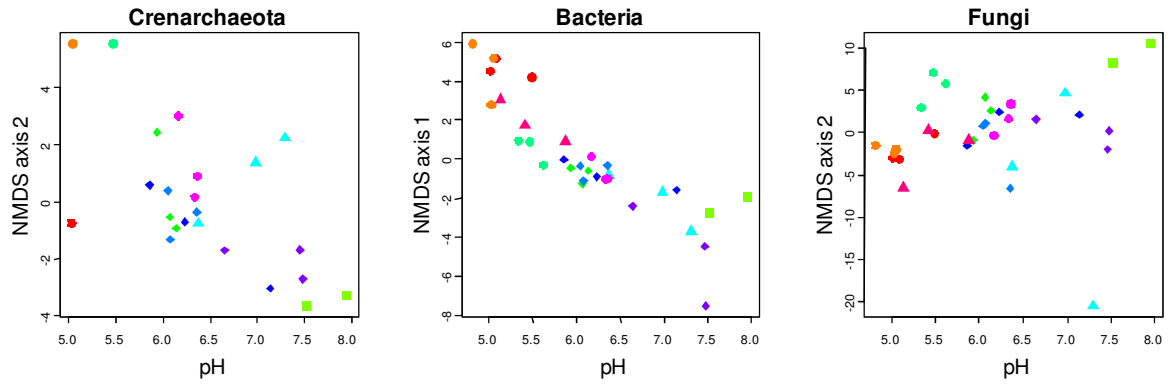

Figure 4: Relationships between CE-SSCP microbial profiles' fitted configuration in NMDS ordination (axis 1 for bacteria, axis 2 for crenarchaeota and fungi) and soil pH. See Table 1 for symbols and code correspondences.

First of all, bacteria do display physiological preferences for particular pH, such as *Acidobacteria* that are often more abundant in low-pH soils (Sait et al., 2006; Lauber et al., 2008; Zinger et al., in press) and of which certain clades better grow on low-pH media (Sait et al., 2006). The large abundance of *Acidobacteria* in soils (Janssen, 2006); their sensibility to pH (Lauber et al., 2008), and the strong co-variation found between soil pH and bacterial communities suggest that the effect of soil pH could be more direct on bacteria as shown by the strongest relation of bacterial similarity patterns with this variable (Table 3). As a consequence, we found that bacterial communities had concordant spatial distribution with plant communities (Table 3). Nevertheless, as aforementioned, soil pH can also integrate other soil variables that we did not measured here. Indeed, bacterial communities displayed however a certain degree of variability between sampling units replicates (Table 2) except in TR and FG. While TR is dominated by a leguminous species that probably promote N_2-fixing bacteria, such as *Rhizobium*, the high linkage between FG plants and bacterial communities remains unexplained.

In this study, we followed-up only the group *Crenarchaeota* of archaea because they have previously been described as the most abundant, widely distributed archaeal group in terrestrial ecosystems, and quite related to rhizosphere (DeLong, 1998). We found here that the spatial distribution of crenarchaeal communities was partially related to those of bacterial

and plant communities, probably due to their co-variation with soil pH (Fig. 4). These results are supported by the fact that we obtained not or rarely PCR product of crenarchaeal 16S rRNA genes in samples from CF, CTR, and FP, of which soil pH are below 6, and the NDMS ordination of crenarchaeal communities was strongly dispersed except in ES and SR, which display soil pH above 7 (Fig. 3, Table 2). This and the results of Mantel tests (Table 3) suggest that soil pH may be a good predictor in the spatial patterns of crenarchaeal communities (Fig. 4) according to the findings of Nicol et al. (Nicol et al., 2008). However, crenarchaeal communities seemed strongly overdispersed between and within plant communities (Table. 2, Fig. 3). This pattern corroborates with the earlier observation of Oline and colleagues (Oline et al., 2006) that observed the dominance of different crenarchaeal groups in subsamples from a same soil core, and hypothesized that crenarchaeal communities displayed discrete and clumped spatial distribution. This implies that the sampling effort in our study was insufficient to characterize the whole crenarchaeal community in a given sampling unit and may explain the dispersed ordination that we observed (Fig. 3).

Fungal communities were also related to soil pH and plant cover, but quite less than prokaryotic communities (Fig. 3, 4, Table 3). This feature can reflect their ability to occur and be highly competitive in soils of which pH are far from their optimum of growth (review in Standing and Killham, 2007). Nevertheless, fungal communities appeared characteristics of plant communities of which soil pH are acidic, and also in KS (Table 2). Moreover, soil fungal communities have already been described as strongly influenced by N addition, which concomitantly caused a soil acidification (Bardgett et al., 1999). Interestingly the spatial distribution of soil fungi was also quite related to live phytomass and slightly to soil organic matter (Table 3). Live phytomass has been previously described as a well suited proxy of the plant above and below-ground net primary productivity in grasslands (Gill et al., 2002; Scurlock et al., 2002). The correlation between fungal communities and live phytomass was also consistent by considering only plant communities dominated by herbaceous vegetation in the Mantel test (Spearman's $\rho = 0.3$, $P < 0.001$). These results suggest that fungal communities in productive meadows differ from less productive ones, supporting previous assumptions (Chapman et al., 2006). Productive systems can reflect higher soil nutrient availability, which have been reported to strongly influence fungal communities' structure (Bardgett et al., 1999; Lauber et al., 2008). These observations coupled with the slight influence of soil organic matter on fungal communities (Table 3) can reflect an effect of soil fertility on the spatial distribution of fungal communities observed here. This hypothesis needs, however, confirmation by assessing the soils C, N or P content in our system.

However, soil pH, live phytomass, and SOM do not fully explain the spatial patterns of fungi observed here (Table 3), and the great variability that fungal communities displayed in a same plant community can reflect the variability of local unmeasured parameters.

Taken together, these results suggest that microbial communities do exhibit similar spatial patterns to plant cover though the plant-soil feedbacks on soil properties, noticeably soil pH, but also on other unknown factors. These uncharacterized factors could vary more locally, which can explain the variability between sampling units replicates. Finally, microbial community structure is slightly related to the diversity of above-ground plant community. We did not assessed microbial diversity since CE-SSCP profiles are not sufficiently accurate to estimate such indices. Nevertheless, it will be of primary interest to estimates microbial diversity though, for instance, pyrosequencing in order to better characterize the relationships between above and below-ground diversity in this fragmented landscape.

Finally, our study also provided indications about the effect of geographic distance on microbial communities at the landscape-scale. It is now increasingly recognized that microbial communities exhibit spatial patterns that can be, to a certain extent, related to those of macro-organisms, such as distance-decay relationship (Green and Bohannan, 2006). However, at a landscape scale, microbial communities do not co-varied with geographic distances in contrast to floristic composition (Table 3). This feature was also true by controlling the environment similarity (by using a partial Mantel test, $P < 0.37$). These results support the previous conclusions of Fierer et al (Fierer et al., 2007), inasmuch as the hypothesis of Baas-Becking seems true at the landscape scale. This feature, however, does not contradict the aforementioned distance-decay relationship argued by Green et al (Green and Bohannan, 2006) given that nothing exclude that two similar habitats separated by long distances and consequently submitted to different past events share the same microbial pool (Hughes Martiny et al., 2006).

CONCLUSION

Microbial communities in alpine soils displayed spatial patterns that mirror the soil pH gradient and the distribution of plant communities at the landscape scale. The apparent effect of soil pH on biotic communities could be the detail that hides a multitude of mechanisms involving many soil parameters such as nutrient availability. Although our experimental design prevents to dissociate the effect of abiotic and biotic factors, it offers an overview of the landscape organization of both plant and microbial communities. The characterization of

other factors responsible of such a pattern could be enhanced by measuring nutrient availability in our system or by integrating the functional traits of dominant plants, or even by decoupling all these confounding environmental variables in controlled conditions.

We also found that microbial communities were not influenced by geographic distance but rather by environmental conditions, supporting the Baas-Becking hypothesis at the landscape scale. Finally, this study calls for more attention of plant cover in any attempt to evaluate the factors responsible of microbial community assembly and diversity at local and regional scales.

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III. Principaux résultats et discussion

1. Gradient d'acidité des sols et distribution de la flore

Le site d'étude montre un important gradient de pH du sol. Ce gradient est fortement dépendant de la végétation. En effet, la roche mère du site d'étude étant de nature basique, les flux de matière organique vers le sol conduisent, dans notre cas, à une acidification des sols par accumulation d'acides organiques. Ce phénomène serait d'autant plus important dans notre site en raison de l'altitude, le recyclage de la matière organique étant réduit par des températures faibles. De même, une végétation dense conduit à une plus grande acidification des sols alors qu'une végétation squelettique présente des sols d'avantage soumis à l'effet de la roche mère. Or, dans notre site d'étude, la densité du couvert végétal est fortement liée à sa composition floristique. De plus, les traits fonctionnels de la végétation déterminent en partie les conditions édaphiques (Eviner and Chapin, 2003). Ainsi, **les communautés végétales s'organisent le long du gradient de pH du sol** selon la densité des groupements, leur composition en espèce mais aussi probablement en fonction des caractéristiques éco-physiologiques des espèces dominant ces communautés. L'ensemble des facteurs suivis, *i.e.* le pH et la teneur en matière organique du sol, la productivité des groupements végétaux et leur composition floristique sont donc **confondants**. Leurs effets respectifs sur la distribution spatiale des communautés végétales et microbiennes ne peuvent donc être déterminés dans notre étude. Celle-ci offre néanmoins l'opportunité de caractériser l'organisation des communautés végétales et microbiennes dans le paysage.

2. Le pH du sol, indicateur des patrons spatiaux microbiens ?

Nos résultats montrent que des sols de pH similaire abritent des communautés microbiennes similaires. Ce résultat est particulièrement vrai pour les bactéries, pour lesquelles le pH du sol montre une corrélation de 68 % avec les patrons de similarité entre les communautés bactériennes. Ce degré de corrélation est tout à fait comparable aux 66 % de corrélation décrits entre le pH et les communautés bactériennes de la matière organique benthique (Fierer et al., 2007a). Les *Acidobacteria* ont précédemment été décrites comme largement répandues dans le sol, représentant en moyenne plus de 20% des communautés bactériennes du sol (Janssen, 2006). Nos précédentes investigations ont montré que ce taxon était prédominant aussi bien dans les sols de situations thermique et nivale (Article C, Annexe

B). Or, ce taxon diminue de façon systématique avec une augmentation du pH (Fierer et al., 2007; Lauber et al., 2008, Article C). L'effet du pH sur les communautés microbiennes pourrait donc être direct, par recrutement de taxons montrant des préférences physiologiques pour des gammes de pH bien définies. Cependant, le séquençage des génomes de trois espèces d'*Acidobacteria* n'a montré qu'une faible représentation de gènes potentiellement impliqués dans la tolérance à des pH acides, suggérant que les organismes appartenant à ce phylum ne sont pas obligatoirement acidophiles (Ward et al., 2009). Des études supplémentaires sont donc nécessaires pour définir le rôle du pH du sol dans l'assemblage des communautés bactériennes.

L'effet du pH a également été retrouvé dans la distribution spatiale des communautés crenarchaeotes, mais dans une moindre mesure (35% de corrélation). Bien que le pH du sol ait été décrit comme peu influant à court terme sur la structure des communautés crenarchaeotes (Nicol et al., 2004), une étude plus récente des mêmes auteurs a révélé un effet significatif de cette variable sur la structure phylogénétique de ces organismes (Nicol et al., 2008). Dans le cadre des différentes études que nous avons pu mener ces trois dernières années, nous avons systématiquement eu beaucoup de mal à amplifier par PCR les gènes l'ADNr 16S des crenarchaeotes lorsque les échantillons montraient un pH acide (ici, CF, CTR, V, FP ; les situations nivales du Chapitre II; et des sols de pelouse dominées par *Carex curvula* ou *Nardus stricta*), suggérant une abondance moindre de ce taxon dans des sols de pH acide. Néanmoins, ces organismes montrent une distribution spatiale en patch, formant des populations clonales très isolées (Oline et al., 2006). Il est donc possible que notre effort d'échantillonnage ait été insuffisant pour détecter les crenarchaeotes dans les sols acides. Cela pourrait également expliquer la co-variation plus faible des crenarchaeotes avec le pH du sol, nos profils moléculaires n'étant peut-être pas suffisamment représentatifs du point d'échantillonnage. Enfin, il est également probable que le pH du sol soit confondant avec, par exemple la teneur et/ou la nature de la matière organique, ou d'autres variables affectant de manière plus significative l'assemblage des communautés que nous n'avons pas mesurées ici.

Cette dernière proposition paraît également applicable aux communautés fongiques. En effet, nous avons également pu observer une co-variation des communautés fongiques et du pH du sol (28%), mais aussi avec la productivité du milieu (32%) et la teneur en matière organique (15%) qui pourraient représenter, dans une certaine mesure, la fertilité des sols. Le pH a été décrit comme régulant la disponibilité de l'azote et du carbone (Kemmitt et al., 2006). De plus, les communautés fongiques sont fortement structurées par la teneur en nutriments dans le sol, notamment en azote et en phosphore (Lauber et al., 2008; Singh et al.,

2009). En parallèle, les champignons sont capables de montrer une grande tolérance à des pH éloignés de leur optimum de croissance (Standing and Killham, 2007). Bien que le pH du sol n'ait pas été rapporté comme facteur influençant les patrons spatiaux des communautés fongiques, les investigations sur le sujet (Kasel et al., 2008; Lauber et al., 2008) ont été menées sur des gradients de pH de moindre amplitude qu'ici (pH de 4.5 à 8). La covariance du pH du sol et des communautés fongiques peut donc être à la fois liée à des préférences physiologiques, mais aussi et surtout à des facteurs covariants avec le pH. Il est donc nécessaire de mieux caractériser les conditions édapho-chimiques, notamment en termes de ressources, pour une meilleure compréhension de la distribution spatiale des champignons. Pour conclure, l'apparente corrélation entre les patrons spatiaux du pH du sol et des communautés microbiennes peut résulter d'un effet rétroactif de l'activité microbiologique.

3. Microbiogéographie à l'échelle du paysage

L'article E a donc révélé une certaine **cohérence de l'organisation du paysage de la microflore** en relation avec le couvert végétale et les conditions édaphiques associées**.** Cependant, les communautés microbiennes ne semblent pas s'organiser selon la distance géographique, suggérant ainsi que l'hypothèse de Baas-Becking, « *everything is everywhere,* but, *the environment selects* », est vrai à l'échelle du paysage. Ainsi, les micro-organismes dans notre site sont potentiellement ubiquistes par des phénomènes de dispersion et d'accumulation de spores ou propagules dans les sols, mais les conditions biotiques et abiotiques recruteraient des espèces différentes dans ce pool d'espèces commun. Ces résultats vont dans le sens de ceux d'une précédente étude à l'échelle du paysage sur les communautés bactériennes (Fierer et al., 2007a) et suggèrent que l'échelle du paysage est insuffisante pour observer une décroissance de similarité entre communautés sous l'effet de la distance géographique (*distance-decay relationship*). Afin de compléter notre étude, nous avons séquencé la région ITS1 de l'ADN métagénomique fongique pour les différents points d'échantillonnage. Ces jeux de données sont en cours d'analyse (en collaboration avec Guillaume Lentendu, Christelle Melodelima, Stéphanie Blanc-Manel, LECA) et les séquences des gènes ADNr 16S bactériens devraient être obtenues courant 2009.

Les communautés microbiennes s'organisent dans le paysage selon les conditions environnementales, et cette organisation peut être prédite dans une certaine mesure par le pH du sol et la composition floristique sus-jacente. Il reste néanmoins à caractériser d'autres facteurs pouvant expliquer de façon plus nette l'organisation spatiale des communautés fongiques.

Discussion et Perspectives

L'objectif de cette thèse était, dans un premier temps, d'optimiser et de développer des outils moléculaires et d'analyse permettant d'appréhender les communautés microbiennes. A l'aide de ces méthodes, nous nous sommes ensuite employés à comprendre quels étaient les mécanismes impliqués dans l'assemblage des communautés microbiennes des sols alpins. Dans ce but, nous avons caractérisé la dynamique de ces communautés dans deux habitats alpins fortement contrastés par leur régime d'enneigement, leur végétation et leur fonctionnement. Notre étude s'est ensuite étendue à une étude paysagère des communautés microbiennes alpines, sous différents couverts végétaux présentant des caractéristiques édapho-climatiques distincts.

I. Les outils d'analyse, puissance et limites

Nous avons pu constater au cours des différentes parties de ce manuscrit qu'il existait des méthodes variées permettant de caractériser les communautés microbiennes, dont certaines sont présentées dans la Fig. 18. Ici, nous nous proposons de faire un état des lieux de la puissance et des limites de l'ensemble de ces méthodes, et de leur pertinence dans des cas variés.

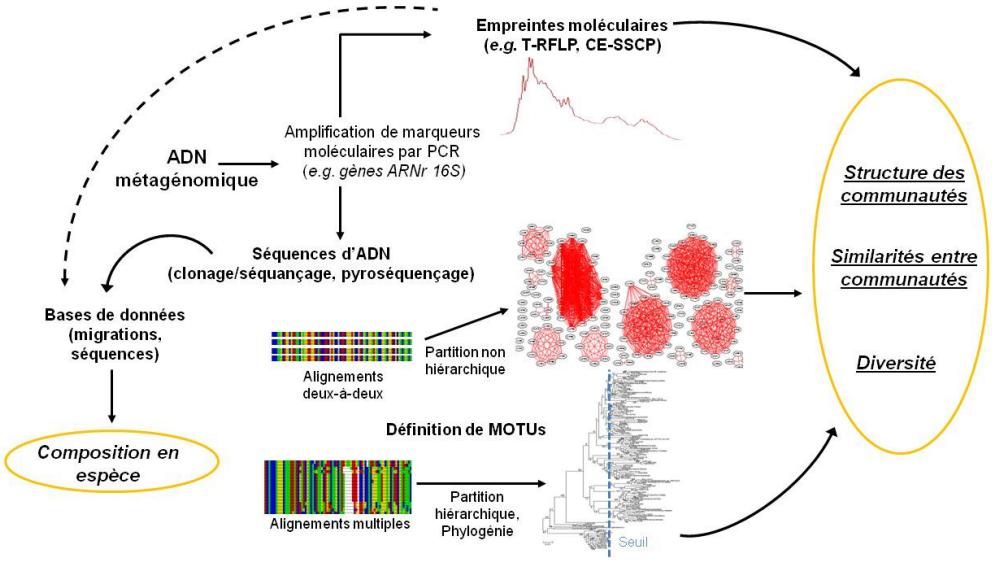

Figure 18 : Les différents outils permettant de caractériser les communautés microbiennes.

1. Bilan sur les techniques d'empreintes moléculaires

Jusqu'à récemment, l'utilisation des techniques d'empreinte moléculaire, comme la SSCP, sur les micro-organismes était limitée au génotypage de souches pathogènes dans un cadre clinique. A l'aide d'une banque de temps de migration des fragments d'ADN obtenus de cultures microbiennes pures, il est en effet possible de déterminer quelle est la souche présente dans un échantillon simple *via* ces méthodes (Fig. 18). Cette application des méthodes d'empreintes moléculaires n'est pas possible sur des matrices plus complexes telles que le sol où la diversité des micro-organismes est bien supérieure. En effet, cette diversité entraîne une saturation du signal traduite par la co-migration de phylotypes taxonomiquement différents. Cette saturation est rapidement atteinte puisqu'il a été montré que l'information était saturante à partir de 35 phylotypes (Loisel et al., 2006). Néanmoins, les méthodes d'empreintes moléculaires, en particulier la CE-SSCP, peuvent tout à fait être appliquées dans le cadre d'études comparatives de matrices complexes (cf. Chapitre I).

L'information semi-quantitative générée par les techniques d'empreinte moléculaire permet d'accéder à la structure des communautés (dominance d'un/plusieurs phylotypes), de les comparer, mais aussi d'estimer leur diversité (Fig. 18). Or, le degré de sensibilité de ces méthodes, en termes de résolution taxonomique, n'est pas connu. Dans le chapitre II, nous avons pu suivre la dynamique saisonnière des communautés bactériennes et fongiques par CE-SSCP et clonage séquençage. A partir de l'identification des séquences dans les bases de données Ribosomal Database Project (http://rdp.cme.msu.edu) et Genbank (http://www.ncbi.nlm.nih.gov), il est possible d'évaluer la similarité des communautés microbiennes à différents rangs taxonomiques et ainsi d'accéder à la résolution taxonomique de la CE-SSCP (Fig. 19). La CE-SSCP a un niveau de sensibilité équivalent à l'ensemble des rangs taxonomique, en particulier à celui du sous-phylum chez les bactéries. De même les distances entre les échantillons obtenues par CE-SSCP sont quasiment identiques à celles obtenues au niveau de l'ordre ou de la famille chez les champignons. Ces résultats indiquent donc que cette méthode est **fiable à des niveaux taxonomiques fins**.

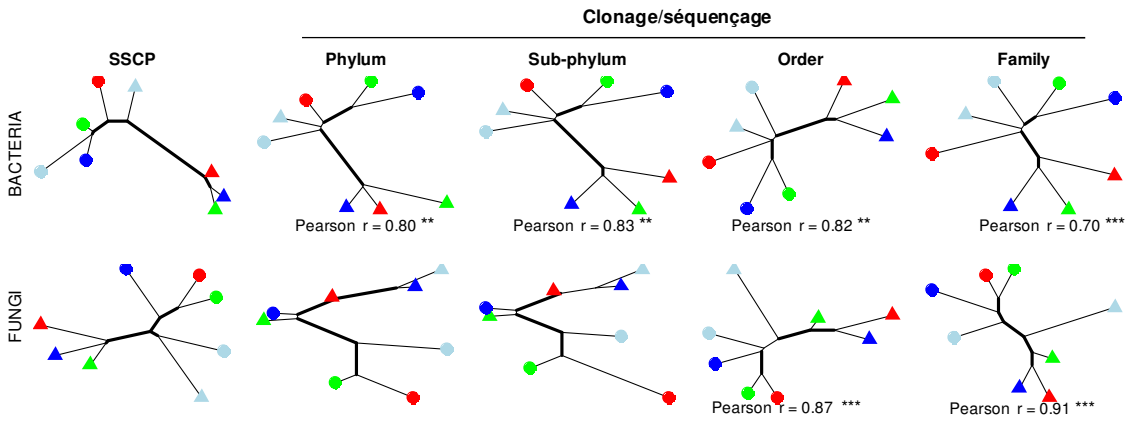

Figure 19 : Résolution taxonomique de la CE-SSCP. Les symboles représentent les situations thermiques (triangle) ou nivales (cercle). Les couleurs représentent les saisons d'échantillonnage : Mai (bleu clair), Juin (bleu), Août (vert), Octobre (rouge). Les branches en gras sont supportées par des valeurs de bootstrapp supérieures à 50% (1000 retirages). Les données sont issues de l'Article C (cf. Chapitre II), basées sur la région V3 du gène de l'ADNr 16S pour les bactéries, et sur l'ITS1 pour les champignons. La corrélation entre les matrices de distances issues de la CE-SSCP est celles obtenues par identification des séquences ont été évaluées à l'aide d'un test de Mantel (1000 retirages). La significativité du test est indiquées de la façon suivante : ** : $P < 0,01$, *** : $P < 0,001$

Généralement, l'information tirée des profils moléculaires se limite à une identification du degré de similitude entre les communautés microbiennes. Or, une étude récente a montré qu'il est possible de tirer beaucoup plus d'informations à partir des profils moléculaires (Loisel et al., 2006). Par exemple, le nombre de pics est proportionnel au nombre d'OTU (Operational Taxonomic Units) dans l'échantillon, permettant d'accéder la **richesse** de la communauté. Par ces méthodes, il est également possible d'estimer la **diversité** des communautés microbiennes de façon fiable en utilisant l'estimateur de Curtis (Curtis et al., 2002). Bien que la validité empirique des indices de diversité dérivés de méthodes d'empreinte moléculaire ait été récemment remise en question (Bent and Forney, 2008), leur utilisation reste néanmoins **valable dans un cadre comparatif**.

L'ensemble de ces observations couplées aux résultats obtenus dans le chapitre I montrent la fiabilité et la haute sensibilité de détection de la CE-SSCP. Les méthodes d'empreintes moléculaires, et en particulier la CE-SSCP donnent donc accès à des informations de qualité, presque semblables à des méthodes plus lourdes comme le séquençage massif. Ainsi, ces méthodes constituent de très bons candidats pour des

applications à haut débit, comme par exemple dans le cadre de contrôle de qualité de matrices complexes telles que le sol ou l'eau en comparaison avec des échantillons de référence.

2. Perspectives pour le séquençage massif

La caractérisation des communautés microbiennes *via* le clonage/séquençage ou le pyroséquençage est une pratique maintenant courante. Les jeux de données obtenus par ces méthodes peuvent apporter des informations qualitatives et semi-quantitatives sur la composition des communautés microbiennes (cf §Introduction II.4). Dans ce contexte, le plus intuitif est d'estimer l'abondance relative des grands groupes microbiens en identifiant les séquences grâce aux bases de données de type Genbank (Fig. 18). Ce type d'approche reste cependant peu résolutif car les groupes sont souvent identifiés à des niveaux taxonomiques élevés (*e.g.* phylum). De plus, la fiabilité des banques de données internationales est douteuse, en particulier pour les séquences fongiques (Nilsson et al., 2006).

Une analyse plus fine des jeux de données de séquences permet de préciser la diversité et l'assemblage des communautés. Classiquement, elle consiste à (i) soumettre les séquences à un algorithme d'alignement multiple, (ii) calculer des distances entre les séquences alignées (iii) effectuer une classification hiérarchique permettant d'évaluer le pourcentage de recouvrement des communautés comparées ainsi que d'estimer leur richesse et leur diversité (Fig. 18). A partir d'arbres phylogénétiques, il est aussi possible d'accéder aux diversités α (intra communauté) et β (écart entre communautés) (Lozupone and Knight, 2008) en s'affranchissant de l'unité de l'espèce (cf. Introduction III.1).

Il est également possible d'évaluer le degré de concordance entre la structure phylogénétique et l'environnement en utilisant des outils tels que le test d'Unifrac (Lozupone et al., 2007) ou le NTI (Nearest Taxa Index, (Webb et al., 2002)). L'information contenue dans la structure phylogénétique des communautés permet de préciser l'importance relative des facteurs écologiques et évolutifs impliqués dans leur assemblage (Martin, 2002; Webb et al., 2002). Cela revient à déterminer si les conditions environnementales favorisent des taxons microbiens phylogénétiquement proches. Nous avons ainsi pu constater que les communautés bactériennes étaient phylogénétiquement proches en automne, ce qui n'était pas visible par l'étude des phyla ou par des approches d'empreintes moléculaires (Annexe B, Article C).

Or, tous les marqueurs moléculaires ne peuvent être soumis à la démarche précédemment citée. En effet, les marqueurs moléculaires hautement polymorphiques en

tailles (comme l'ITS1) ne peuvent être alignés correctement avec des algorithmes d'alignement multiples (Loytynoja and Goldman, 2008). La méthode que nous proposons dans l'Article D (cf. Chapitre II) permet de contourner cette limitation en utilisant des algorithmes d'alignements deux-à-deux et des méthodes de classification non hiérarchiques (Fig. 18). L'utilisation de cette méthode d'analyse rend tout à fait possible l'estimation du degré de similarité entre plusieurs communautés, de leur diversité ou de leur richesse.

Dans le cadre de traitement de données issues du pyroséquençage, l'utilisation d'un algorithme exact est extrêmement coûteuse en temps. Cette approche est donc en cours d'optimisation dans le but de réduire ces temps de calculs (Guillaume Lentendu, Christelle Melodelima, Eric Coissac, LECA). Une autre limitation danse notre démarche d'analyse est de ne pas établir de lien hiérarchique entre les clusters. En utilisant cette méthode, il est donc difficile d'observer les effets potentiellement filtrant de l'environnement sur la structure phylogénétique des communautés microbiennes. Dans ce but, il serait envisageable de reconstruire une hiérarchie entre les clusters (Fig. 20). Cette démarche consisterait classer nos différents groupes par taxon, par exemple par sous-phylum, puis pour chacun d'entre eux, (i) de générer une séquence « résumée » de chaque cluster, (ii) d'évaluer la similarité entre ces séquences résumées et (iii) reconstruire une hiérarchie entre les clusters (Fig. 20). Il est cependant probable que cette méthode ne soit pas applicable aux marqueurs moléculaires trop polymorphiques en taille de par l'utilisation d'alignements multiples. Ces développements seront effectués sous la direction d'Eric Coissac (LECA).

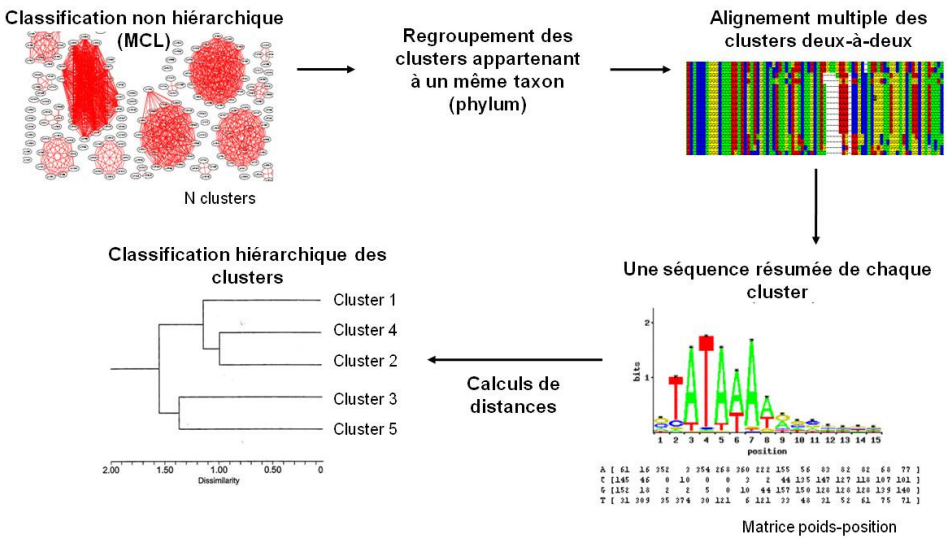

Figure 20 : Développement possible pour la démarche d'analyse proposée dans l'Article D.

3. L'ambigüité des espèces rares

Les micro-organismes constituent la majeure part de la diversité et de la biomasse des êtres vivants (cf. Introduction I.1). Avec l'avènement des techniques moléculaires, l'enjeu principal en microbiologie environnemental a été de quantifier de manière exacte cette immense diversité (Hughes et al., 2001; Curtis et al., 2002; Quince et al., 2008). Dans ce souci d'exhaustivité, une batterie d'estimateurs ont été développés parmi lesquels on distingue deux catégories principales : la raréfaction et les estimateurs de richesse (*e.g.* Chao1 et ACE, revue dans Hughes et al., 2001). Brièvement, la raréfaction estime le nombre d'espèces dans un échantillon pour un nombre d'individus donné. En prenant en compte un nombre croissant d'individus et en effectuant des retirages aléatoires, il est possible de déterminer les écarts de richesse entre échantillons. Cette méthode diffère des estimateurs de richesse, tels que Chao1 ou ACE, qui calculent la richesse totale d'un échantillon en estimant les espèces rares totales à partir du nombre d'espèces rares observées (revu dans Hughes et al., 2001). Ces espèces rares représentent effectivement une part majeure dans la diversité des communautés microbiennes (Sogin et al., 2006).

Cependant, la signification biologique des espèces rares est ambiguë. Premièrement, il est difficile de savoir si ces espèces sont biologiquement actives, ou s'il ne s'agit pas de traces « fossiles », résultantes de l'accumulation de spores dans le sol (Sogin et al., 2006). Il est donc difficile de statuer sur l'importance fonctionnelle qu'occupent ces espèces rares, sachant de plus que chez les macro-organismes, les espèces abondantes sont responsables de la majeure part du fonctionnement de l'écosystème (revue dans Grime, 1998). Deuxièmement, leurs effectifs et leur présence/absence dans un échantillon sont fortement liés à l'effort d'échantillonnage, tant bien sur le terrain que pendant les phases d'extraction d'ADN, de PCR, ou de séquençage (Curtis et al., 2002; Quince et al., 2008).

Dans le chapitre II, l'assemblage des communautés microbiennes des systèmes thermiques et nivaux présentait une grande diversité d'espèces rares probablement responsable leur dynamique saisonnière. Nos résultats montraient également que les deux systèmes étudiés présentaient un pool d'espèces microbiennes commun, mais se différenciaient fortement par l'abondance de ces espèces (Articles C, D, Annexe B). De même, les résultats obtenus dans le chapitre III suggèrent fortement qu'à l'échelle du paysage, les sols de différents habitats disposent également d'un pool d'espèces commun. Ainsi, dans le cadre d'étude comparative de communautés microbiennes soumises à différentes conditions environnementales mais issues d'une même province (*sensu* Hughes Martiny et al., 2006),

l'utilisation d'estimateurs de richesse paraît futile. Aux vues de ces observations, l'utilisation d'indices de diversité moins sensibles aux espèces rares, comme l'inverse de l'indice de Simpson, apparaissent être la meilleure alternative, même si les abondances relatives des espèces peuvent être biaisées par les étapes de PCR ou séquençage (Bent and Forney, 2008).

La présence et l'importance des espèces rares dépend du passé écologique de l'écosystème, et pourraient constituer un réel réservoir de diversité génétique et fonctionnel (Sogin et al., 2006). Le nombre d'espèces constituant ce pool serait donc déterminant dans la résilience des écosystèmes suite à une perturbation. Ainsi, nous avons pu observer dans le chapitre II que l'assemblage des communautés microbiennes différait selon les saisons, mais aussi d'une année à l'autre suite à la période hivernale (Article C). L'estimation de la richesse totale des communautés microbiennes permettrait donc d'évaluer la magnitude de la plasticité des communautés microbiennes ainsi que le potentiel de résilience d'un écosystème. Ce type d'estimation est donc nécessaire dans le contexte actuel de pollutions chroniques et de changements globaux.

Ainsi, la **prise en compte des espèces rares dépend de la question biologique**. Dans le cadre d'études comparatives des communautés microbiennes d'une même province ou de leur réponse à court terme à une variable environnementale, il convient mieux d'utiliser des indices de diversité. L'estimation de la richesse est quant à elle, nécessaire sur des études menées à long terme ou à plus grande échelle.

4. Importance des méthodes classiques

Bien que les outils moléculaires présentent l'avantage d'accéder aux micro-organismes non-cultivables, ces méthodes indirectes ne donnent accès qu'à une information semi-quantitative, de part les biais survenant aux étapes d'échantillonnage et de manipulation de l'ADN (cf. Introduction II.4). Elles ne permettent donc pas d'estimer de façon empirique la biomasse microbienne. Or, cette variable est un indicateur de la qualité des sols et de la quantité de carbone immobilisée par les communautés microbiennes. Certains l'assimilent même à une mesure de la disponibilité des ressources (Waldrop et al., 2006). Plus particulièrement, le ratio biomasse fongique/biomasse microbienne (F/B) renseigne particulièrement sur leur productivité et la fertilité de leurs sols. En effet, les bactéries dominent les sols fertiles alors que les champignons constituent la majeure part de la biomasse microbienne dans des sols plus pauvres de zones tempérées (Wardle et al., 2004).

Dans le cadre des chapitres II et III, nous avons effectué des dénombrements bactériens et des mesures de biomasse microbienne par fumigation extraction. Ces mesures devaient êtres misent en relation avec les données de profils moléculaires ou de séquençage de façon à mieux caractériser les facteurs responsables des variations spatiales et temporelles de la densité et de l'assemblage des communautés microbiennes. Malheureusement, ces données se sont révélées inexploitables pour des raisons diverses (contaminations de produits, etc…). Il aurait été également souhaitable d'accéder au ratio F/B de façon à mieux identifier le fonctionnement de nos sites, mais nous ne disposions pas des outils nécessaires à cette mesure.

L'isolement des souches microbiennes et les observations microscopiques constituent également une approche complémentaire à l'étude des communautés microbiennes. En effet, bien que les techniques basées sur la biologie moléculaire donnent accès aux micro-organismes non cultivables, elles présentent l'inconvénient de produire des inventaires majoritairement composés d'espèces inconnues. Par exemple, la plupart des phylotypes fongiques de situation nivales obtenus dans le chapitre II demeurent bien mal caractérisés (Article D). L'isolement de souches microbiennes ou de structures d'association microbiennes/racinaires (Fig. 21), identifiées par des approches systématiques et par séquençage des gènes ribosomaux microbiens permettrait de constituer une base de données de qualité, spécifique à nos sites d'échantillonnage. Cette base de données pourrait dans une certaine mesure combler le manque d'informations des banques de clones, et pourrait aussi préciser le degré de relation des phylotypes majoritaires avec la rhizosphère.

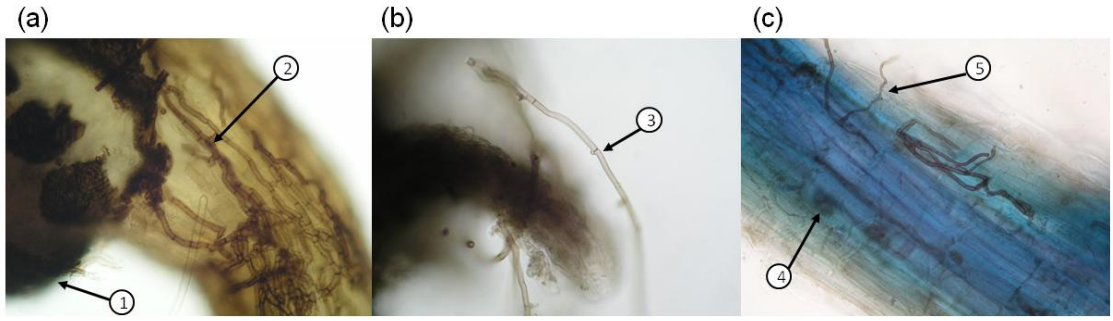

Figure 21 : Observation microscopiques de champignons associés aux racines. (a) Racine de situation nivale : 1/structure ectomycorhizienne, 2/ mycélium d'endophyte racinaire (dark septate fungi, DSE). **(b)** racines joubarbe (*Sempervivum sp.)* de situation thermique : 3/mycélium bouclé d'un basidiomycète non identifié. **(c)** racine de *Festuca paniculata* :4/ endomycorhize à arbuscule vésiculaire, 5/ mycélium d'endophyte racinaire (DSE) sortant de la racine. *Photographies : L. Sage.*

Dans le contexte du chapitre II, l'inventaire des souches cultivable a été effectué chez les bactéries (Bahar Shahnavaz, LECA) et les champignons (Lucile Sage, LECA). La confrontation des banques de clones obtenus dans à ces inventaires font l'objet d'une collaboration avec Lucile Sage, Claire Molitor, Marie-Audrey Quintama et Bello Mouhamadou (LECA) pour les communautés fongiques, et sont en cours de finalisation chez les bactéries (Shahnavaz B., Gury J., Geremia R.A., in prep.). Enfin, ces inventaires ont permis de mettre en évidence des espèces psychrophiles, qui pourraient montrer certaines activités enzymatiques d'intérêt dans un cadre biotechnologique.

5. Lien entre composition et fonction des communautés microbiennes

Les méthodes utilisées au cours de cette thèse permettent de caractériser les communautés microbiennes d'un point de vue phylogénétique. Bien que ces approches permettent d'identifier les taxons présents dans les communautés et les facteurs responsables de leurs structures, elles n'apportent que peu de renseignements sur le rôle des micro-organismes dans l'écosystème. Dans ce contexte, il existe plusieurs alternatives permettant de lier la structure phylogénétique à la structure fonctionnelle des communautés microbiennes (Table 3).

Premièrement, il est possible de caractériser la capacité d'utilisation de substrats (*e.g.* SIGR, BIOLOG) et les activités enzymatiques de souches isolées (Table 3, Torsvik and Ovreas, 2002; Prosser, 2007). La détection de production ou résistance aux antibiotiques peut également renseigner sur la compétitivité de la souche. Cependant, ces méthodes ne permettent pas de déterminer la fonction qu'occupe la souche dans son environnement. En effet, les performances métaboliques de la souche *in situ* peuvent être influencées par ses interactions avec les autres organismes (*e.g.* compétition, inhibition de croissance) ou par les conditions environnementales. Par exemple, une même espèce de champignon mycorhiziens peut exercer des activités métaboliques extrêmement variables selon son milieu (Buee et al., 2007).

Enfin, bien que les techniques d'isolement connaissent des développements significatifs permettant d'accéder à des souches jusqu'alors non cultivables (Cardenas and Tiedje, 2008), elles demeurent inaptes à isoler la majorité des micro-organismes. Néanmoins, cette approche demeure la plus fiable dans la caractérisation des fonctions et des potentiels biotechnologiques des micro-organismes. L'ensemble de ces techniques de criblage serait tout

à fait envisageable sur les inventaires de cultivables isolés des échantillons provenant du chapitre II.

Les autres techniques sont basées sur la biologie moléculaire (Table 3) et peuvent être divisées en trois catégories. Le premier type de méthodes consiste à étudier les gènes ribosomaux et en parallèle un ou plusieurs gènes fonctionnels d'intérêt (sous forme d'ADN ou d'ARNm) comme cela a été proposé pour les gènes impliqués dans la nitrification ou la dénitrification (revu dans Bothe et al., 2000) ou ceux codant pour la laccase, une enzyme extracellulaire impliquée dans la dégradation de composés carbonés complexes (Luis et al., 2004). Les microarrays reposent sur ce principe et sont plus exhaustives par criblage d'un grand nombre de gènes fonctionnels. Ces techniques présentent cependant le désavantage de ne suivre que des gènes déjà connus, et il est possible que les gènes ciblés ne représentent qu'une fraction minoritaire des gènes fonctionnels présents dans l'échantillon (Cardenas and Tiedje, 2008).

Table 3 : Exemples d'approches permettant d'établir un lien entre la composition et la fonction des communautés microbiennes du sol. *D'après Prosser, 2007.*

Approches	Description	Avantages	Inconvénients
Etudes physiologiques de souches isolées	Tests enzymatiques et suivi d'utilisation de substrats par photométrie ou fluorométrie	Potentiel métabolique des souches Avancées biotechnologiques.	Ne reflète pas les conditions *in situ*. Pas de renseignement sur les non cultivables.
Empreinte moléculaire ou clonage/séquençage de gènes fonctionnels à partir d'extraits d'ADN ou d'ARNm	Basé sur la PCR, approche ciblée.	Estimation de l'abondance des gènes ciblés (ADN ou ARNm). Estimation du nombre de taxons capables d'assurer la fonction (ADN ou ARNm). Estimation du niveau d'expression des gènes et donc de l'activité métabolique (ARNm).	Restreint à des gènes connus. N'indique pas si les produits géniques sont fonctionnels *in situ*.
Microarray	Criblage à haut débit des gènes présents dans l'échantillon par hybridation de l'ADN ou ARNm.	Permet d'analyser simultanément les taxons et les gènes fonctionnels présents.	Restreint à des gènes connus
Métagénomique ou Métatranscriptomique	Séquençage massif de l'ensemble de l'ADN ou de l'ARNm.	Permet d'accéder à des fonctions inconnues. Information sur l'abondance relative des grands groupes taxonomiques leur activité métabolique. Peut caractériser de voies métaboliques d'organismes non-cultivables.	Grande majorité des séquences de gènes ribosomaux. Nombreuses séquences non assignées.
Utilisation d'isotopes stables (SIP)	Utilisation de sources de carbone radio-marqué assimilable dans l'ADN, l'ARN, les acides phospolipidiques ou les protéines	Mise en évidence directe des liens entre la fonction et l'organisme responsable de cette fonction. Observation des réseaux trophiques.	Limité à des substrats carbonés Information potentiellement noyée par les réseaux trophiques.

Avec l'avènement des techniques de séquençage à haut débit, la métagénomique, basée sur l'ADN, permet de contourner cette limite en criblant l'ensemble des gènes présents dans l'échantillon. En comparant et réassemblant les séquences obtenues par rapport à des génomes de références, il est possible (i) de caractériser la structure phylogénétique des communautés microbiennes (gènes ribosomaux), (ii) d'identifier les gènes fonctionnels par homologie aux génomes de référence et (iii) d'identifier de nouvelles fonctions et (iv) de reconstruire des génomes d'organismes non cultivables. La métatranscriptomique permet quant à elle d'identifier les ARNm présents dans l'échantillon et donne donc accès au niveau d'expression des gènes fonctionnels (Prosser, 2007; Cardenas and Tiedje, 2008). Ce type d'approche est actuellement en cours de développement au laboratoire (Jean-Marc Bonneville, Tarafa Mustafa, Armelle Monier, LECA) dans l'optique de caractériser la fonction des micro-organismes des écosystèmes thermiques et nivaux, dont la structure phylogénétique a précédemment été caractérisée dans le chapitre II.

La demande croissante d'une caractérisation de la diversité et de la nature des fonctions présentes dans un sol a fait naître une nouvelle perspective pour la microbiologie environnementale : une approche basée sur les traits fonctionnels. Ce type d'approche est de plus en plus utilisé pour l'étude des macro-organismes, en particulier chez les plantes (Lavorel and Garnier, 2002). En effet, certaines caractéristiques morphologiques, biochimiques ou éco-physiologiques chez les végétaux reflètent la performance d'une espèce ou le type de fonction qu'elle peut occuper dans l'écosystème, tel que le taux de croissance (*e.g.* surface foliaire, longueur des racines) ou la décomposabilité (*e.g.* teneur en matière sèche ou rapport carbone/azote dans les feuilles).

L'un des challenges majeurs en écologie microbienne est donc d'identifier des traits microbiens donnant accès à leur fonction dans l'écosystème. Ces traits peuvent être mesurés *via* l'ensemble des méthodes précédemment citées, par exemple par mesure des activités enzymatiques d'isolats. A partir de techniques plus récentes, certains auteurs ont proposé le nombre de gènes de synthèse d'antibiotiques, indiquant le degré de compétition dans l'environnement, ou celui des gènes impliqués dans la motilité, indiquant une plus grande compétitivité pour les ressources (revu dans Green et al., 2008a). De telles approches pourraient ainsi permettre une meilleure compréhension du fonctionnement des écosystèmes et de l'écologie de ces organismes. Les deux écosystèmes contrastés étudiés dans le chapitre II seraient par exemple de bons modèles pour tester les approches basées sur les traits sur communautés microbiennes, et pourrait en contre partie nous renseigner de façon significative sur les processus biogéochimiques se déroulant dans ces habitats.

II. Facteurs régissant l'assemblage des communautés microbiennes

Les chapitres II et III ont permis de mettre en évidence de forts contrastes dans la distribution spatiale et temporelle de la composition des communautés microbiennes alpines. Cette hétérogénéité résulte de mécanismes complexes s'opérant entre les communautés microbiennes et les conditions environnementales. L'ensemble de ces processus a une influence sur l'assemblage des communautés microbiennes à court et long terme, que nous nous proposons de discuter ici.

1. Des effets à court terme: dynamique des communautés microbiennes

Les conditions édapho-climatiques peuvent varier sur des pas de temps relativement courts. Premièrement, les conditions climatiques fluctuent tout au long de l'année, particulièrement dans les écosystèmes alpins, soumis à d'importantes amplitudes saisonnières (notamment thermique et hydrique), elles-mêmes nuancées par les gradients mésotopographiques et d'exposition à l'ensoleillement (Körner, 1995). Dans le chapitre II, nous avons ainsi pu mettre en évidence un effet significatif de l'amplitude de la saisonnalité, en particulier chez les bactéries de situation thermique (Fig. 22, Article C). En hiver, les températures du sol chutent à des températures nulles ou négatives selon l'épaisseur du manteau neigeux. Ces hétérogénéités de températures hivernales pourraient générer des différences entre les communautés microbiennes. Cependant, nous avons observé un effet filtre des conditions édapho-climatiques hivernales sur l'assemblage des communautés microbiennes, recrutant des espèces communes dans les deux habitats étudiés (Fig. 22, Chapitre II).

L'hiver coïncidant avec une équilibration des pH de sol thermiques et nivaux, il est difficile de déterminer qui de la température, du pH du sol, ou de l'absence d'activité végétale est responsable de cet événement sélectif. En effet, ces deux derniers facteurs sont déterminant dans l'assemblage des communautés microbiennes (Kowalchuk et al., 2002; Fierer and Jackson, 2006). L'assemblage des communautés hivernales peut donc résulter (i) d'un recrutement d'espèces similaires pour des températures nulles et négatives, (ii) de l'acidification des sols de situation thermique soit par lessivage du à la présence d'un fin manteau neigeux, soit par enrichissement des sols suite à la dégradation de la matière organique par des champignons psychrophiles (Annexe C), ou (iii) l'absence de végétation et

par conséquent d'exsudation racinaire, excluant les espèces dépendantes de ces ressources ou (iv) une complémentarité de ces effets. De telles hypothèses restent néanmoins à vérifier par des expérimentations *in situ* et en laboratoire, notamment *via* l'étude mise en place dans le cadre de l'Annexe C qui a également été effectuée sur des sols de systèmes nivaux, par une acidification artificielle des sols ou encore par élimination du couvert végétal.

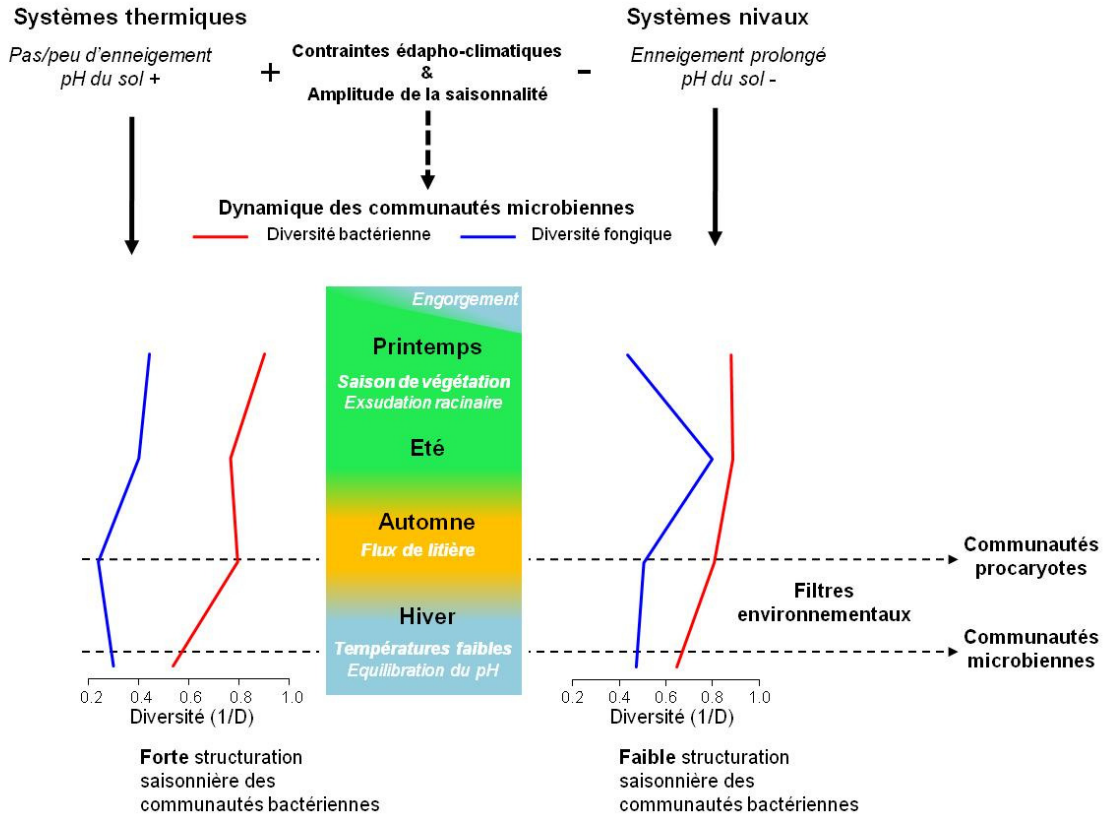

Figure 22 : Synthèse de l'effet à court terme des régimes d'enneigement sur la microflore. Les régimes d'enneigements influencent la dynamique saisonnière des communautés microbiennes du sol, représentée ici en terme de diversité (inverse de l'indice de Simpson : 1/D). La dynamique des communautés bactériennes est très structurée dans des systèmes à forte amplitude saisonnière. Les conditions hivernales constituent un événement majeur qui tend à recruter des taxons bactériens et fongiques similaires dans les deux situations. En automne, les conditions environnementales favorisent certains taxons procaryotes.

Les rythmes saisonniers et les gradients d'enneigement déterminent la densité de peuplement et la phénologie de la végétation. Celle-ci constitue le principal apport de la matière organique dans le sol par renouvellement et exsudation des racines durant la saison de végétation et par dépôt de litière en phase de sénescence (Eviner and Chapin, 2003). En conséquence, la disponibilité et la qualité des ressources dans le sol varie avec l'avancement

de la saison de végétation. Dans ce sens, les résultats du chapitre II indiquent clairement une succession des communautés microbienne dont la diversité varie durant la saison de végétation (Fig. 22). De tels résultats ont déjà été observés sous différentes espèces végétales (Duineveld et al., 2001; Mougel et al., 2006; Singh et al., 2007). Plus particulièrement, Mougel et al. (2006) ont mis une évidence un effet significatif du passage de la plante au stade reproductif sur les communautés microbiennes. La dynamique des communautés microbienne peut donc aussi être expliquée par une dynamique de la rhizodéposition en termes de qualité et/ou quantité ou du taux de renouvellement des racines au cours du stade de développement.

Enfin, le dépôt de litière en automne enrichit le sol en matière organique complexe. Ce phénomène semble promouvoir des décomposeurs bactériens, et recrutent des espèces particulières chez les crenarchaeotes (Fig. 22). Cette tendance semble moins franche pour les champignons, bien qu'on observe une recrudescence de phylotypes affiliés au genre *Inocybe* en situation nivale (Article D), dont le statut saprophyte est établi. Ces mêmes organismes se retrouvent tout au long de l'année en situation thermique, formant des associations ectomycorhiziennes avec *Dryas octopetala* (Article D). Le suivi des activités enzymatiques de cette espèce en situation thermique nous renseignerait sur la dynamique de son statut mutualiste ou saprophyte, et il est fort probable, aux vues de nos précédents résultats, que ce dernier apparaisse en automne.

L'assemblage des communautés microbiennes est donc extrêmement **sensible aux variations à court terme de températures, de pH du sol, et de disponibilité des ressources** (Berg and Smalla, 2009). Cependant, l'effet réciproque des micro-organismes sur les variations des conditions édaphiques demeure inconnu, et pourrait faire l'objet de recherches supplémentaires par exemple par transplantation de sols de situation thermique en situation nivale et inversement, couplé à une mesure des nutriments.

Enfin, les fluctuations saisonnières des conditions édapho-climatiques apparaissent déterminantes sur la diversité β des communautés bactériennes (Annexe B). Dans un contexte de réchauffement global, une modification des rythmes saisonniers pourrait, à terme, profondément affecter cette diversité. Bien que nous n'ayons pas établi de relation entre ce rythme saisonnier et la diversité β des communautés fongiques, les effets du réchauffement global pourraient également avoir un impact significatif sur l'assemblage de ces communautés et potentiellement négatif sur la diversité des champignons observée en situation nivale (Fig. 22).

2. Relation entre communautés bactériennes et fongiques

Les bactéries et les champignons constituent la majeur part de la biomasse des décomposeurs du sol. Leurs interactions sont donc potentiellement déterminantes dans le fonctionnement des écosystèmes. Or, la majorité des études se concentrent sur les bactéries, certaines ciblent les champignons, et une minorité sont portées sur ces deux règnes microbiens. Cette thèse avait pour objectif de suivre à la fois la dynamique saisonnière et les patrons spatiaux des communautés fongiques et bactériennes. Dans le chapitre II (Article C) et le chapitre III (Article E), nous avons pu constater une réponse similaire de la structure des communautés fongiques et bactérienne aux contraintes environnementales, comme par exemple le couvert végétale, le pH du sol, ou les conditions hivernales (Chapitre II et III). Ceci implique que l'assemblage des communautés fongiques et bactériennes est régi par des variables similaires. En parallèle, l'étude menée dans le cadre du chapitre II a révélé un **comportement antagoniste de la diversité** de ces deux règnes tant bien dans le temps qu'en rapport avec le couvert végétal (Fig. 22).

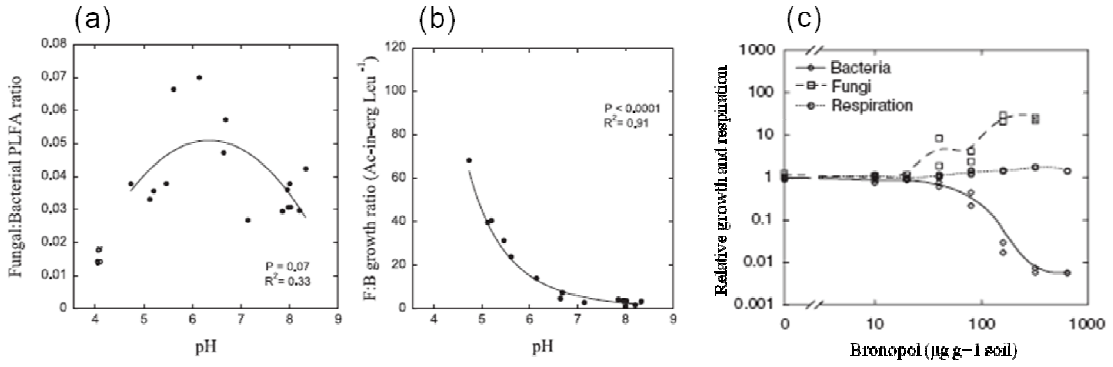

Figure 23 : Antagonisme de la biomasse et de la croissance bactérienne et fongique. (a) Relation du ratio de biomasse fongique/bactérienne avec le pH du sol. **(b)** Relation entre le ratio de croissance fongique/bactérienne avec le pH du sol. Ces résultats sont issus de terrains agricoles soumis à une histoire de traitement des terres commune. *D'après Rousk et al. (2008).* **(c)** Effet de l'inhibition de la croissance bactérienne sur la croissance fongique et sur l'utilisation des ressources. *Source : Rousk et al. (2009).*

Quelle est l'implication écologique de cet antagonisme ? L'équipe d'Erland Bååth a extensivement étudié la co-variation de la biomasse et la croissance bactérienne et fongique en relation avec différentes variable édaphiques, notamment le pH du sol (Fig. 23). Ces études ont montré une relation antagoniste du ratio de biomasse fongique et bactérienne pour des pH de valeurs supérieures à 6 (Fig. 23a). Cet antagonisme se traduit également au niveau de la

croissance (Fig. 23b). En outre, la croissance des champignons est fortement favorisée en présence d'inhibiteurs de la croissance bactérienne, sans pour autant changer l'utilisation des ressources (Fig. 23b). Ces résultats suggèrent un recouvrement des niches écologiques de ces organismes (Rousk et al., 2008).

Nous avons précédemment évoqué une dominance de la biomasse bactérienne en système fertile (Wardle et al., 2004), qui peut être attribuée à une plus grande compétitivité des bactéries pour les composés solubles, notamment par la production d'inhibiteurs de croissance fongique ou de facteurs séquestrant les nutriments (de Boer et al., 2005). Aux vues de ces observations, il est possible que les patrons de diversité mis en évidence dans le chapitre II reflètent une compétition pour les ressources, traduite par une disponibilité et/ou une diversité de niches supérieure en situation nivale. C'est dans ce contexte que des mesures de ratio de biomasse fongique/bactérienne auraient constitué une information complémentaire sur l'interaction entre ces deux règnes. Dans la situation actuelle de réchauffement climatique, de diminution de la biodiversité, ou dans un contexte appliqué en agronomie, il pourrait s'avérer nécessaire de déterminer l'existence d'une relation entre la biomasse microbienne et sa diversité et éventuellement les facteurs responsables d'une telle relation.

3. Des effets long terme : relation entre le couvert végétal et les communautés microbiennes

La présence de racines affecte fortement l'assemblage des communautés microbiennes du sol (Kowalchuk et al., 2002; Singh et al., 2007). En effet, contrairement au sol, la rhizosphère constitue un milieu propice au développement des micro-organismes par sa richesse en nutriments et les nombreux échanges possibles entre les populations microbiennes (Standing and Killham, 2007; Berg and Smalla, 2009). En outre, la rhizosphère a tendance à acidifier les sols par libération d'acides organiques contenus dans les exsudats racinaires (Eviner and Chapin, 2003). Ces observations confortent les résultats obtenus dans le cadre de l'étude du chapitre III, montrant que l'assemblage des communautés dépend fortement du pH des sols, dont les patrons spatiaux découlent de la densité du couvert végétal (Fig. 24). Mais les relations entre le compartiment végétal et le compartiment microbien vont bien au de là.

Certaines espèces végétales sont régulièrement associées de façon positive ou négative avec certains organismes du sol (Berg and Smalla, 2009). Par exemple, nous avons observé une prédominance de champignons ectomycorhiziens (*Inocybe*, *Russula*) dans les systèmes thermiques, largement décris comme étant associés aux plantes dominantes de ces sites

(*Kobresia myosuroides*, *Dryas octopetala*) (Article D). De même, nous avons remarqué une forte similarité des communautés bactériennes de pelouses à *Trifolium pratense* (légumineuse) qui peut être attribuée à la présence d'espèces nodulantes telles que *Rhizobium* (Article E). Dans ce sens, (Saetre and Baath, 2000) ont montré que la distribution spatiale des communautés bactériennes dépendait de la position des arbres appartenant à différentes espèces. Ainsi, il semble que la distribution spatiale des espèces végétales co-varie avec celle des micro-organismes. Cette relation pourrait aussi être liée à la qualité de la matière organique associée aux différentes espèces végétales (Saetre and Baath, 2000).

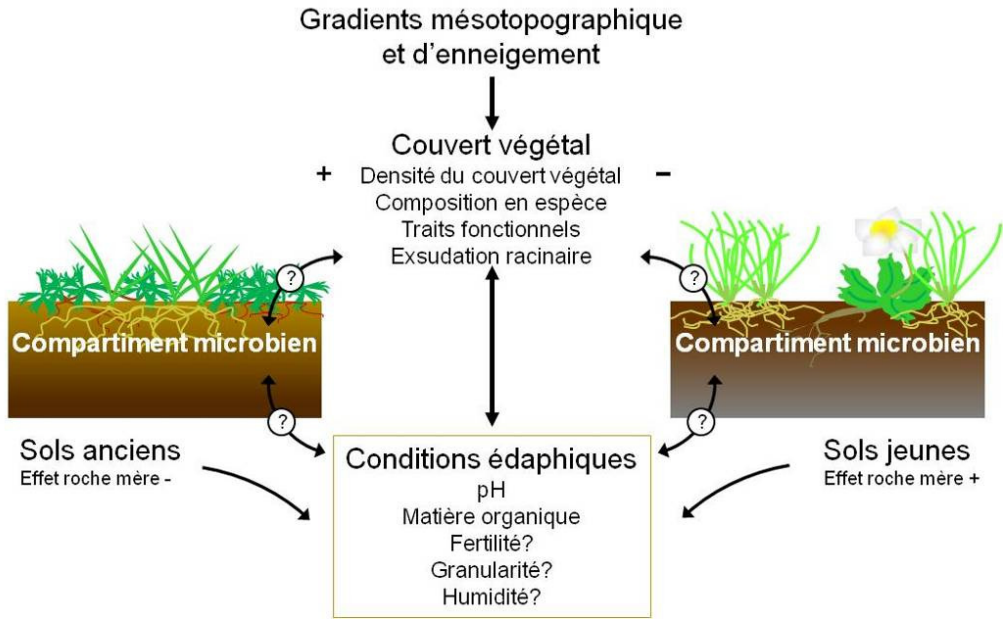

Figure 24 : Facteurs responsables de l'organisation paysagère des communautés microbiennes.

Les espèces végétales se distinguent par leur contenu foliaire en carbone ou autres métabolites ainsi que dans la qualité ou quantité d'exsudation racinaire. Ces différences éco-physiologiques ont un impact significatif sur les conditions édaphiques (*e.g.* pH, matière organique Eviner and Chapin, 2003). Les communautés végétales suivies dans les chapitres II et III sont dominées par des espèces différant dans leur écophysiologie (Choler, 2005; Baptist, 2008). Leur distribution spatiale a un impact significatif sur l'assemblage des communautés microbiennes (Article E). La distribution spatiale des communautés microbiennes pourrait donc résulter de l'écophysiologie des espèces végétales, notamment les espèces dominantes puisqu'elles constituent la majeure part de la biomasse (Grime, 1998).

La relation entre les traits fonctionnels végétaux et l'assemblage des communautés microbiennes demeure obscure. En effet, bien que (Bardgett et al., 1999) ait montré un effet significatif des traits fonctionnels sur les communautés microbiennes, d'autres études soutiennent un effet prépondérant des conditions édaphiques (Singh et al., 2009). Or, ces deux facteurs sont intimement liés (Eviner and Chapin, 2003), ce qui peut expliquer la corrélation existant entre les communautés fongiques et la biomasse végétale, « proxy » de la fertilité des sols (Article E). De même, d'autres études menées en microcosmes ont révélé une relation entre la structure des communautés microbiennes et certains traits foliaires (Singh et al., 2009), Bousaria A., Mustafa T., Geremia R.A., Choler P., en préparation). Les **traits fonctionnels végétaux** semblent donc agir **indirectement** sur l'assemblage des communautés microbiennes, en contribuant au façonnement des sols (Fig. 24).

Ainsi, dans les chapitres II et III, nous avons pu observer une certaine **cohérence entre les patrons spatiaux végétaux et microbiens**. Cette relation résulte de long processus environnementaux dont le plus important apparait être l'état d'avancement de la formation des sols. Ces processus sont complexes et font intervenir une multitude de facteurs confondants dont les interactions demeurent bien mal caractérisés (Fig. 24) et leur caractérisation constitue en soi un vaste champ de recherches. Dans notre cas, il serait nécessaire de mesurer d'autres variables édaphiques comme la teneur en azote dissous ou en phosphates, ainsi que la granulométrie des sols, dont l'influence sur les communautés microbiennes a précédemment été rapportée (Kasel et al., 2008; Lauber et al., 2008).

Enfin, la nature de la relation entre la diversité végétale et la diversité du compartiment microbien reste mal caractérisée (Hooper et al., 2000). Alors que certaines études ont mis en évidence ce type de relation entre les communautés fongiques et végétales (revu dans Berg and Smalla, 2009), d'autres n'ont pas observé de telles relations (Waldrop et al., 2006). Les phylotypes bactériens et fongiques issus de l'étude du chapitre III sont en court de séquençage dans le but de caractériser le type de correspondance entre la diversité végétale et microbienne, mais aussi dans le but de préciser l'impact des facteurs édaphiques sur cette diversité (en collaboration avec Guillaume Lentendu, Stéphanie Manel et Christelle Melodelima).

4. Perspective : vers une microbiogéographie

Nous avons mis en évidence une **organisation paysagère des communautés microbiennes** déterminée par des mécanismes complexes s'opérant entre le compartiment microbien, la végétation et les conditions édapho-climatiques (cf. Chapitres II et III). Les données du chapitre III ont révélé l'absence de corrélation entre l'assemblage des communautés microbiennes et la distance géographique à l'échelle du paysage. Ce résultat sera vérifié avec des données de pyroséquençage. A l'échelle du paysage, nous avons donc pu mettre en évidence un effet déterminant de l'habitat sur l'assemblage des communautés microbiennes. A une échelle sensiblement supérieure, (Fierer et al., 2007a) ont obtenu des résultats similaires, suggérant qu'a l'échelle du paysage, l'hypothèse de Baas-Becking « *everything is everywhere*, but, *the environment selects* » est valable. Cependant, de telles études où tous les facteurs sont confondants ne permettent pas de déterminer quelle est la part de chacune des variables environnementales dans la distribution spatiale des espèces microbiennes.

A plus grande échelle, il semble que les communautés microbiennes montrent une dissimilarité croissante avec la distance géographique (Green and Bohannan, 2006). L'ensemble des études sur le sujet n'a cependant pas pris en compte la dissimilarité des habitats (cf. Introduction §III.2). Dans l'optique de préciser l'hypothèse de Baas-Becking à plus grande échelle, nous avons initié, sous l'impulsion de Philippe Choler (LECA), un suivi du compartiment microbien à plus grande échelle comprenant l'ensemble du massif des Alpes et la partie méridionales des Carpates (Fig. 25). Ce suivi s'est concentré sur 3 espèces végétales présentées dans la Fig. 25, et pourrait donc également permettre de préciser la part respective des variables édaphiques sur l'assemblage des communautés microbiennes. Nous avons dans un premier temps effectué utilisé la CE-SSCP dans le cadre de cette étude, dont nous ne décriront pas les résultats dans le présent manuscrit.

Une autre étude, incluse dans un projet collaboratif sur la dynamique et le fonctionnement des populations et communautés biotiques de falaises d'altitude (projet ECOVER, LECA), visera à étendre nos connaissances sur la distribution spatiale des communautés microbiennes. L'étude se portera sur des micro-écosystèmes de falaises générés par *Silene acaulis*, une espèce formant des coussinets, où la matière organique du sol résulte de la dégradation de sa litière. Ce sont donc des systèmes extrêmes et clos où les flux populationnels sont probablement limités. La caractérisation du compartiment microbien dans de tels écosystèmes permettrait de préciser l'influence de l'isolement des communautés

microbiennes sur leur distribution spatiale, ainsi que d'apporter des indications sur leur fonctionnement.

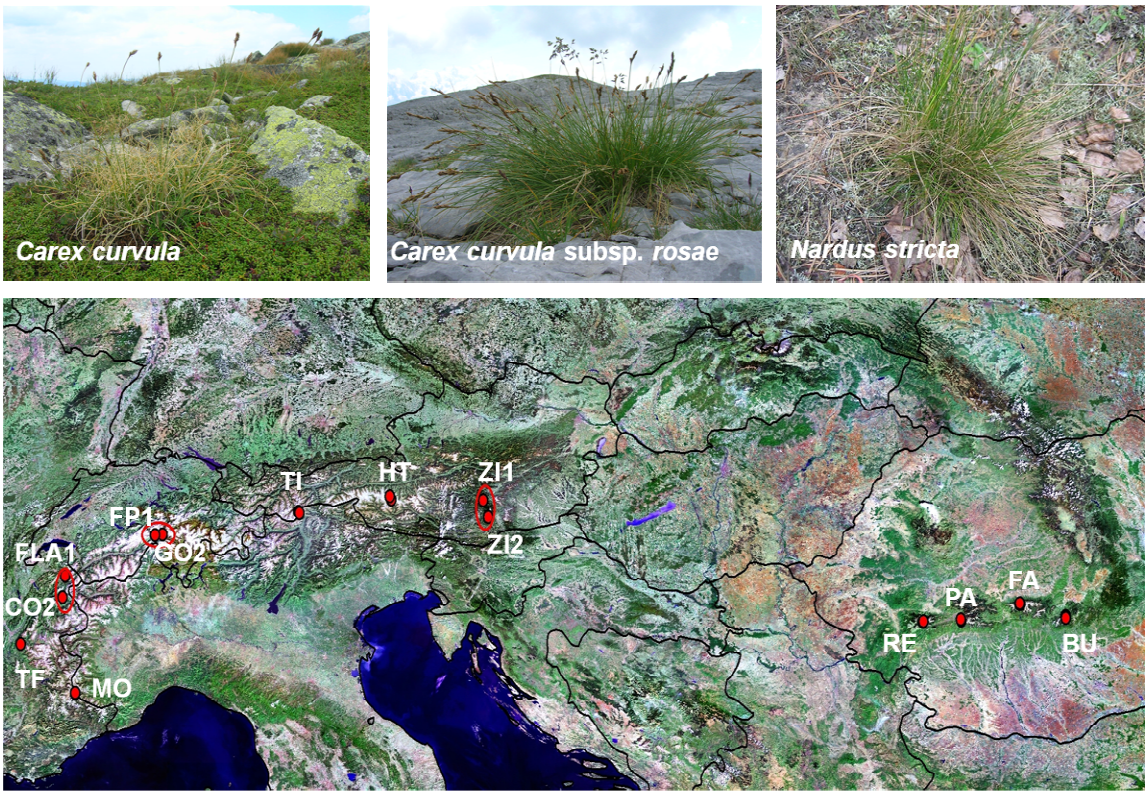

Figure 25 : Présentation des espèces végétales suivies et de la distribution des points d'échantillonnage. La zone de prélèvement s'étend sur l'ensemble de l'arc Alpin (à gauche) et la zone méridionales des Carpates méridionales (à droite). *Source: P.Choler.*

Enfin, nous avons remarqué un renouvellement des communautés microbiennes d'une année à l'autre suite à la perturbation que constitue l'hiver (Article C). Ce renouvellement doit néanmoins être confirmé par des données de séquençage, dont les analyses sont actuellement en cours. La signification écologique d'une telle dynamique demeure peu claire, mais il est possible que ce renouvellement n'ait pas d'incidence sur le fonctionnement de l'écosystème du à la redondance fonctionnelle potentiellement large que peuvent montrer les micro-organismes (Nannipieri et al., 2003). Néanmoins, un nombre croissant d'étude semble montrer que cette redondance fonctionnelle n'est pas valable, et que les communautés microbiennes ne sont pas aussi résilientes qu'attendu aux perturbations (revu dans Allison and Martiny, 2008), ce qui pourrait expliquer le renouvellement interannuel de l'assemblage des communautés suite à la perturbation hivernale observé dans l'article C. Un suivi à plus long

terme de l'assemblage des communautés microbiennes permettrait donc d'évaluer la cyclicité de ce phénomène et de mieux comprendre ses effets ou ses réponses face aux changements globaux. Enfin, s'il s'avère que les changements des communautés microbiennes suite à la période hivernale s'opèrent à des niveaux taxonomiques élevés, et que ce phénomène survient dans d'autres écosystèmes, les études de microbiogéographie devront intégrer de telles dynamiques.

Conclusion

Cette thèse s'est intéressée à l'assemblage et la dynamique des communautés microbiennes et aux techniques permettant d'accéder à cet assemblage. Avec l'actuel développement que connaissent ces techniques et la prise de conscience croissante de l'importance des micro-organismes dans le fonctionnement des écosystèmes, cette thèse appelle à une utilisation des techniques d'analyse des communautés microbiennes en tenant compte de leurs limitations et de la pertinence de leur utilisation en regard des questions biologiques posées. De même, les patrons spatiaux et l'importante dynamique des communautés mis en évidence dans ce travail soulèvent le poids crucial que peut avoir la stratégie d'échantillonnage pour l'étude de la biogéographie des micro-organismes.

Ce travail montre de façon évidente que les régimes d'enneigements sont indirectement responsables de la distribution spatiale et de la dynamique des communautés microbiennes du sol à l'étage alpin. Plus directement, ces patrons spatio-temporels sont une résultante de mécanismes complexes s'opérant entre les compartiments aériens et souterrains. Cette thèse renseigne ainsi sur l'écologie des communautés microbiennes, et appelle à une plus grande prise en compte de la fragmentation du paysage dans le cadre d'études d'impact d'un gradient environnemental sur leur diversité et leur assemblage. Enfin, ces travaux soulèvent la nécessité d'une d'intégrer la dynamique et l'hétérogénéité spatiale des communautés microbiennes aux modèles de processus écosystémiques à l'étage alpin, en particulier dans un contexte de réchauffement global.

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Annexes

Liste des annexes:

Annexe A: Gury J., Zinger L., Gielly L., Taberlet P., and Geremia R.A. (2008) Exonuclease activity of proofreading DNA polymerases is at the origin of artifacts in molecular profiling studies. *Electrophoresis* **29**: 2437-2444.

Annexe B: Shahnavaz B., Zinger L., Lavergne S., Choler P., Geremia R.A.: Snow cover dynamics and phylogenetic structure of bacterial communities in alpine tundra soils. *submitted in Environ. Microbiol.*

Annexe C: Baptist F., Zinger L., Clement J.C., Gallet C., Guillemin R., Martins J.M.F. Sage L., Shahnavaz B., Choler P., Geremia R.A. (2008) Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach. *Environ. Microbiol.* **10**: 799-809.

Annexe A

Exonuclease activity of proofreading DNA polymerases is at the origin of artifacts in molecular profiling studies.

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Research Article

Exonuclease activity of proofreading DNA polymerases is at the origin of artifacts in molecular profiling studies

CE fingerprint methods are commonly used in microbial ecology. We have previously noticed that the position and number of peaks in CE-SSCP (single-strand conformation polymorphism) profiles depend on the DNA polymerase used in PCR [1]. Here, we studied the fragments produced by *Taq* polymerase as well as four commercially available proofreading polymerases, using the V3 region of the *Escherichia coli rss* gene as a marker. PCR products rendered multiple peaks in denaturing CE; *Taq* polymerase was observed to produce the longest fragments. Incubation of the fragments with T4 DNA polymerase indicated that the 3′-ends of the proofreading polymerase amplicons were recessed, while the *Taq* amplicon was partially +A tailed. Treatment of the PCR product with proofreading DNA polymerase rendered trimmed fragments. This was due to the 3′–5′ exonuclease activity of these enzymes, which is essential for proofreading. The nuclease activity was reduced by increasing the concentration of dNTP. The Platinum® *Pfx* DNA polymerase generated very few artifacts and could produce 85% of blunted PCR products. Nevertheless, despite the higher error rate, we recommend the use of *Taq* polymerase rather than proofreading in the framework for molecular fingerprint studies. They are more cost-effective and therefore ideally suited for high-throughput analysis; the +A tail artifact rate can be controlled by modifying the PCR primers and the reaction conditions.

Keywords:
Biodiversity / Capillary electrophoresis-single-strand conformation polymorphism / Environmental microbiology / Microbial communities / Multiple peaks
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1 Introduction

Electrophoretic methods are widely used for molecular profiling of environmental microbial communities [2, 3]. Marker genes are amplified by PCR; the complex PCR product is subsequently separated using electrophoretic methods such as SSCP, denaturing gradient gel electrophoresis (DGGE), or fragment length analysis (FLA). The resulting profiles can then be analyzed by CE coupled to fluorescence detection. Because of its relatively reduced run length, increased sensitivity, and the possibility it affords of parallel runs higher standardization, this method is well suited for high-throughput studies in environmental microbiology.

Molecular profiling relies on the amplification of genetic markers from metagenomic DNA using universal primers that amplify the target genes from a wide variety of different organisms, *i.e.*, the 16S RNA gene (*rss*) for prokaryotic communities. Indeed, the CE-SSCP was recently used for molecular profiling of microbial communities with this marker [1, 4–10]. SSCP is based on conformation of ssDNA, and this conformation depends in turn on the ssDNA sequence. The different conformers are then separated by native electrophoresis [11]. The PCR reaction is key for the reproducibility of this method. However, PCR of polymorphic markers is known to suffer from stochastic and nonstochastic biases [12, 13]. We have recently found that the profile and the number of peaks depend upon which DNA polymerase is used [1]. This difference complicates

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Abbreviations: FLA, fragment length analysis; **MW,** molecular weight

result analysis and profile comparison. In several studies using SSCP or DGGE, the occurrence of closely migrating peaks for isolated strains were reported [1, 10, 14]. These multiple peaks were attributed either to the presence of several conformers for a single sequence [8, 15], or to *Taq* DNA polymerase artifacts (see below) [16, 17]. In fact, increases in resolution and sensitivity have led to the detection of artifacts previously neglected.

Thermostable DNA polymerases are key for PCR amplification. The availability of many natural and modified enzymes is convenient for specific applications. There are two kinds of thermostable DNA polymerases, based on the fidelity of the DNA synthesis: the *Taq*-like and the proofreading DNA polymerases. The thermostable *Taq*-like polymerases belong to the class A DNA polymerase (including the *E. coli* Pol I), but lack the ExoI, ExoII, and ExoIII domains that are involved in 3′–5′ exonuclease activity and which ensure fidelity of the synthesis [18, 19]. Consequently, they exhibit a relatively low fidelity [20] with an error rate ~10 times higher than the proofreading *Pfu* enzyme [21–23]. Depending on the primer sequence, these enzymes also create 3′ adenine overhangs (+A artifact or +A tail) [24]. Adenylation was found to be responsible for the formation of multiple peaks or shorter peaks that can confuse electrophoregram interpretation when analyzing microsatellite polymorphism [16] or conformation polymorphism [17, 25].

The influence on the fingerprint of Class B (*E. coli* Pol II) thermostable proofreading DNA polymerases, like *Pfu* or *Tli*, has not been thoroughly studied. Their proofreading activity depends on a 3′–5′ exonuclease activity that removes mispaired bases of the neo-synthesized strand [20]. Kunkel [26] has reported that the average base substitution error rates of proofreading enzymes range from 10^{-6} to 10^{-7}. Exonuclease activity of these enzymes causes 3′–5′ primer degradation, which prevents their use in SNP detection or in mutagenesis strategies. Because of their fidelity, they are preferred in applications that require a minimal errors in the amplicons, such as gene amplification for protein expression. To limit the formation of artificial ribotypes, they are used for the profiling of microbial communities [4, 7]. Also *Pfu* DNA polymerase was proposed for CE-SSCP because it should provide blunt-ended PCR products, which would avoid interference from random adenylation [24, 25].

We have previously observed that the molecular profiles differ with the kind of DNA polymerases used, and that the proofreading DNA polymerase Isis™ produced multiple closely migrating peaks for a single strain using CE-SSCP [1]. Although ssDNA migration in CE-SSCP depends on its sequence, it also depends on its length. The amplicons were thus analyzed only for their lengths using denaturing (FLA) CE. We also studied the influence of the DNA polymerase on the size of the amplicons. We have found that amplicons obtained with proofreading DNA polymerases are not blunted, but present non-negligible quantities of 3′-end recessed fragments, due to the 3′–5′ exonuclease activity. These are responsible for the shorter peaks.

2 Materials and methods

2.1 Bacterial strains and DNA isolation

Escherichia coli S17-1 was grown aerobically in Luria broth (LB) medium (Qbiogen, MP Biomedicals, Illkirch, France) with strong shaking. 10^9 cells of overnight culture were harvested and washed twice in fresh water. Total genomic DNA was extracted with a Fast DNA kit (Qbiogen) according to the manufacturer's recommendations. DNA concentration was estimated using the Picogreen® DNA quantification kit (Invitrogen, Molecular probes, Cergy Pontoise, France).

2.2 PCR assays

The V3 region of the 16S rDNA gene (*rss*), corresponding to a 205 bp fragment on an *E. coli* genome sequence (http://www.genome.wisc.edu/sequencing/k12.htm), was used as a diversity marker. The bacterial primers used for PCR were HEX-W49 (5′-ACGGTCCAGACTCCTACGGG-3′) [6] and 6-FAM-W104 (5′-TTACCGCGGCTGCTGGCAC-3′) [9, 27]. PCR amplifications were done with the various DNA polymerases listed in Table 1 in an automatic thermocycler GeneAmp, PCR system 9700 (Applied Biosystem, Courtaboeuf, France). The PCR mixture contained 5 ng of DNA template and PCR amplifications were performed according to the manufacturer's recommendations. PCR conditions were 30 cycles of 15 s at 95°C, 15 s at 56°C, 15 s at 72°C and a final extension time of 7 min at 72°C [1]. PCR products were purified with Qiaquick PCR purification (Qiagen, Courtaboeuf, France) as described by the provider. The amplification products were resolved on a 3% agarose gel and stained with ethidium bromide. To test 3′-end modifications, 40 ng of PCR products were also incubated with the DNA polymerases of the study. After heat activation (if needed, see Table 1), the temperature was decreased to 50°C to allow DNA renaturation and fixed at 72°C for 15 min. Reaction mixtures were obtained according to the manufacturers' prescriptions, but dNTP concentrations were 0, 10, 50, and 500 μM in 25 μL final volume.

Table 1. DNA polymerases used in the study

DNA Polymerase	Origin	Proof-reading activity	Manufacturer
Ampli*Taq* Gold[a)]	*Thermophilus aquaticus*	−	Applied Biosystem
Isis	*Pyrococcus abyssi*	+	Qbiogene
Phusion	*Pyrococcus* like	+	Finnzymes
ProofStart[a)]	*Pyrococcus* like	+	Qiagen
Platinum *Pfx*[a)]	*Pyrococcus* sp. KOD	+	Invitrogen

a) Hotstart DNA polymerases need 5–10 min at 95°C for activation.

Treatments with T4 DNA polymerase (New England Biolabs), DNA polymerase I Large Fragment (Klenow, New England Biolabs) and PCR terminator End repair kit (Lucigen Corporation, Euromedex, France) were performed with 40 ng of purified PCR products according to the manufacturer's recommendations in 25 μL reaction volume.

2.3 CE-SSCP

One microliter of 50-fold diluted PCR products was mixed with 10 μL of Hi-Di formamide (Applied Biosystem), 0.5 μL of 0.3 M NaOH and 0.2 μL of internal DNA molecular weight (MW) standard Genescan-400HD ROX (Applied Biosystem). Samples were denatured at 95°C for 5 min and immediately cooled on ice. CE-SSCP was performed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystem) using a capillary 36 cm in length. The nondenaturing polymer consisted of 5% Genscan polymer, 10 % glycerol, and 1 × Tris–borate–EDTA (TBE) (Euromedex, France). Running buffer consisted of 10% glycerol and 1 × TBE and injection time and voltage were set to 22 s and 6 kV. Electrophoreses were performed at 32°C; data were collected for 25 min.

2.4 CE-FLA

In order to check the apparent size of PCR products, denaturing capillary electrophoreses were performed on our ABI Prism 3130xl Genetic analyzer. One microliter of 100-fold diluted PCR products was mixed with 10 μL of Hi-Di formamide and 0.1 μL of internal DNA molecular marker Genescan-500HD Rox. Apparatus skills were 36 cm length capillary, POP4 Genescan denaturing polymer, 60°C for electrophoresis temperature.

2.5 Data analysis

GeneMapper software version 4.0 (Applied Biosystem) was used to analyze all runs in nondenaturing and denaturing migration following the microsatellite-default method. The Genscan-400HD ROX and -500HD ROX MW standard peaks were assigned, respectively, either a pseudo-MW or their actual MW to align the data. The actual MW is the size, in base, based upon a comparison with the profile of Genscan-500HD ROX standards, under denatured conditions. The pseudo-MW is the size arbitrarily expressed in base under native conditions compared to Genscan-400HD ROX standards.

3 Results

3.1 Effects of DNA polymerase type on amplicon size

The CE-SSCP was proposed as a tool for molecular profiling of bacterial communities, on the assumption that a single ribotype will produce a single peak. We have previously found that amplification of 16S RNA gene V3 region of single bacterial species produce two closely migrating peaks, rather than the expected single peak [1], a phenomenon which does not significantly change with migration temperature (data not shown). Since all V3 regions of *E. coli* are identical, the second peak cannot be due to sequence polymorphism. The presence of two conformers for a single DNA sequence is one possible explanation for this effect [8]. To test this hypothesis, the PCR amplicon obtained with Isis DNA polymerase was analyzed by CE-SSCP (Fig. 1A) and CE-FLA. Unexpectedly, several peaks were also detected in CE-FLA: two for the 5′-FAM-W104-labeled strand (201 and 202 bp) and three for the HEX-W49 strand (199, 200, and 201 bp) (Fig. 1B). The areas of the minor peaks obtained with CE-FLA correspond to those observed with CE-SSCP, strongly suggesting that they represent the same fragments. Taken together, these results indicate that the double peak observed in CE-SSCP corresponds to PCR amplicons of different nucleotide length.

We have also studied the size of the amplicons produced by other commercial proofreading DNA polymerases, namely ProofStart™, Phusion™, and Platinum *Pfx* with a classical *Taq* polymerase (Ampli*Taq* Gold®) as a control (Fig. 2A). The Ampli*Taq* Gold produced two well-resolved peaks of 202 and 203 bp for the strand FAM-W104; the

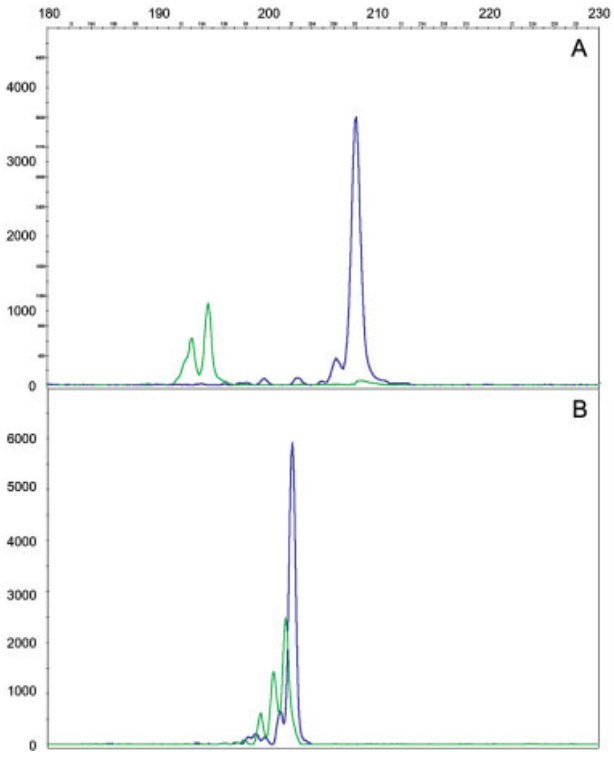

Figure 1. CE-SSCP (A) and CE-FLA (B) profiles of the V3 region of *E. coli* S17-1 amplified with Isis DNA polymerase. Strand HEX-W49 is green, strand FAM-W104 is blue.

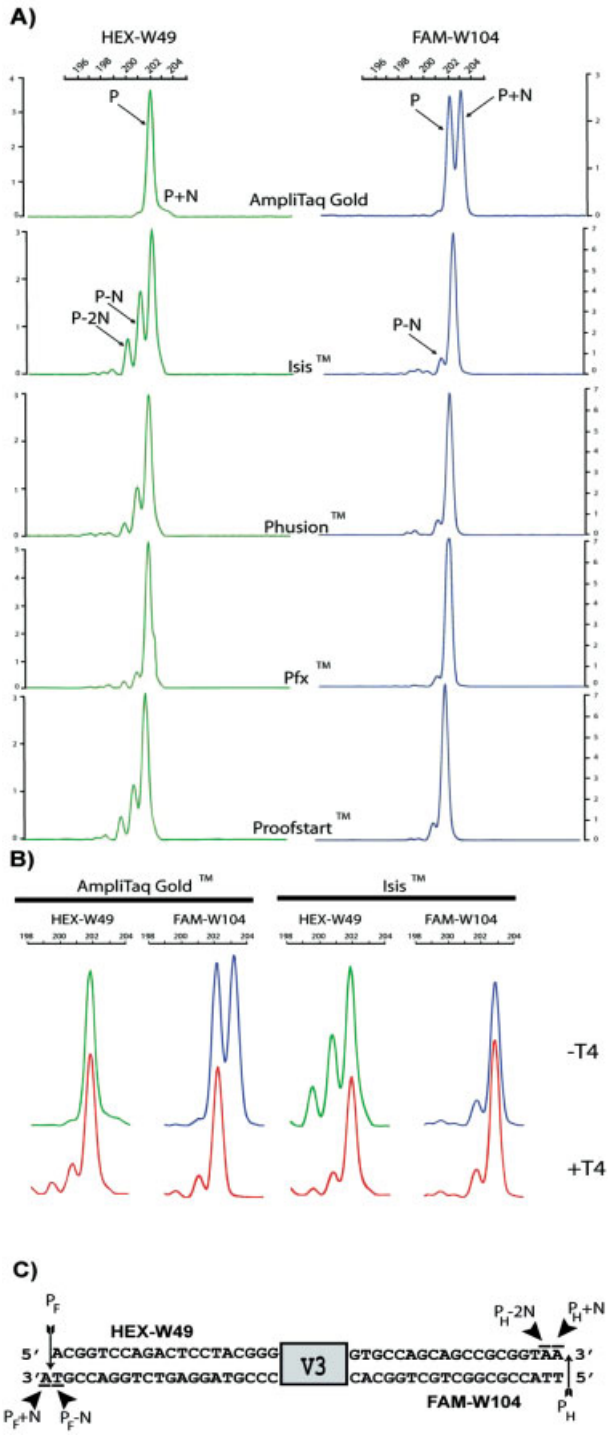

Figure 2. CE-FLA profiles of the V3 region of *E. coli* S17-1. (A) PCR performed with several thermostable DNA polymerases. Fluorescence is expressed in 10^3 rfu unit. (B) Amplicons obtained with Ampli*Taq* Gold and Isis DNA polymerases treated or not with T4 DNA polymerase. (C) Proposed attribution of 3′-end modifications to the obtained peaks. Green and blue profiles are as in Fig. 1, red correspond to samples treated with T4 DNA polymerase. Suffix F and H refer to FAM- and HEX-strands. The underlined bases are missing (for –N or 2N) or added (+N). For the P peaks the arrow indicates the end of the strand.

strand HEX-W49 exhibits one clear peak and a smear running ahead (Fig. 2 A). Meanwhile, the three proofreading enzymes produced three peaks ranged from 200 to 202 bp for the strand HEX-W49 and two sharp peaks of 201 and 202 bp for the strand FAM-W104, but differed in the relative heights of their peaks (Table 2). The comparison of the aligned CE profiles (Fig. 2A) and peak mobility values indicated that the largest Isis amplicon (202 bp) is of similar size of the smallest Ampli*Taq* Gold amplicon (202 bp). The numbers of peaks are almost the same in CE-SSCP and CE-FLA. The change in a single base addition or removal affects the migration in SSCP (see below). Consequently we used CE-FLA because it better reflects single nucleotide differences.

3.2 Modifications inducing size variation

The polymerases studied here modified the 3′-end since the fluorophore, located in 5′-end, was always detected. The best known 3′-end modification of PCR products is the +A-tail generated by *Taq*-like DNA polymerases [17, 24]. However, this activity has not been precisely described in the literature on proofreading DNA polymerases. To elucidate the modification of PCR fragments, we used the T4 DNA polymerase for its 3′ overhang removal and 5′ overhang fill-in capabilities [28]. For the amplicon produced by Ampli*Taq* Gold, the T4 DNA polymerase treatment eliminated both the 203 bp peak of the strand FAM-W104 and the smear of the strand HEX-W49 (Fig. 2B). This result indicates the removal of a base, which most probably corresponds to the +A-tail, (called P + N) and that the remaining peak represents the blunt amplicon (peak P). The T4 DNA polymerase treatment of the Isis amplicon resulted in a decrease of smaller sized fragments and an increase of 202 bp fragments (Fig. 2B) as compared to the other proofreading polymerases tested here (Table 1). Thus, the T4 DNA polymerase filled in recessed 3′-end termini. Consequently, the smaller peaks (Fig. 2A) corresponded to 3′-ends recessed fragments lacking 1 (P − N) or 2 (P − 2N) bases (see Fig. 2C for the modifications of the PCR fragments). T4 treatment increased blunted PCR fragment yield except for Platinum *Pfx*, in which it decreased (Table 3).

We observed the same results with CE-SSCP analysis as in CE-FLA, except that the lower peaks of the W49 strand remained difficult to resolve (see Supporting Information Fig. S1). The one-base strand reduction observed in denaturing condition also led to major peak mobility value displacement and the disappearance or apparition of peaks in CE-SSCP analysis.

3.3 Mechanisms implied in the amplicon size reduction

One possible explanation for the shorter PCR products generated by proofreading polymerases is that the 3′-end nucleotide undergoes a modification preventing further

Table 2. Denaturing CE analysis of W49-W104 PCR fragment obtained with various DNA polymerase with (+T4) or without T4 (−T4) DNA polymerase treatment

DNA polymerase	Peak	Mobility value[a)]				Relative peak area[b)]			
		W49 strand		W104 strand		W49		W104	
		−T4	+T4	−T4	+T4	−T4	+T4	−T4	+T4
Ampli*Taq* Gold	−N	nd	200.52	nd	201.5	–	15.75	–	12.96
	B	201.58	201.57	202.11	202.18	100	84.25	48.73	87.04
	+A	nd	nd	203.17	nd	–	–	51.27	–
Isis	−2N	199.35	199.36	nd	nd	11.8	3.22	–	–
	−N	200.53	200.52	201.06	201.13	30.85	13.09	8.61	13.33
	B	201.58	201.65	202.20	202.25	57.35	83.69	91.39	86.77
Phusion	−2N	199.35	199.28	nd	nd	5.21	2.05	–	–
	−N	200.53	200.43	201.14	201.04	22.01	12.33	7.92	10.59
	B	201.67	201.64	202.19	202.25	72.78	85.62	92.08	89.41
ProofStart	−2N	199.36	199.36	nd	nd	8.36	3.17	–	–
	−N	200.52	200.52	201.13	201.05	22.36	15.45	7.87	14.94
	B	201.65	201.57	202.25	202.18	69.28	81.38	92.13	83.06
Platinum *Pfx*	−2N	199.36	199.28	nd	nd	2.7	2.48	–	–
	−N	200.52	200.44	201.12	201.05	7.48	11.29	5.12	9.78
	B	201.64	201.57	202.25	202.09	89.82	86.23	94.88	90.22

–N, blunt form minus one base pair; nd, no detectable; B, blunt form; +A, A-overhang form; −2N, blunt form minus two base pairs.
a) Mobility values are expressed in base.
b) Relative peak areas are percentage of area of each peak of total area of all peaks.

nucleotide addition. This was tested by incubating the Isis-amplicon with Ampli*Taq* Gold in the presence of a range of dNTP amount (0–500 μM). In the absence of dNTP, the DNA fragment was not modified whereas increased concentrations of dNTP led to a shift of the FAM-W104 strands toward the 202 and 203 bp (data not shown). This indicates that the strands were completed by a +A-tail and that the 3′-end nucleotide was not chemically modified.

The other two possible explanations for the inability of Isis and other proofreading DNA polymerase to produce fully blunted amplicons are: (i) an intrinsic inability to add nucleotides close to the end or (ii) 3′–5′ exonuclease activity. To determine which of these possibilities is true, we have incubated purified *Taq*-amplicons in the presence or the absence of the proofreading DNA polymerase Isis. As expected, in the absence of Isis the product was not modified (Figs. 3A and A'). The addition of proofreading DNA polymerases resulted in a severe trimming of the dsDNA (Fig. 3B). This degradation was particularly extensive for the FAM-W104 strand for which peaks of significant height were found down to 130 bp, whereas the trimming was more limited for the HEX-W49 strand, the major fragments of which were larger than 194 bp. These results indicate the presence of an exonuclease activity related to the Isis polymerase. Extensive degradation was prevented by the presence of dNTP (Figs. 3C–E and C'–E'). In fact, the addition of 10 μM dNTP drastically reduced the extensive degradation of FAM-W104 strand, although shorter strands were still observed. For the HEX-W49 strand, the largest peak was 198 bp. When using 50 μM dNTP, the profiles look like those obtained in manufacturer's recommended conditions. Finally, application of 500 μM dNTP resulted in the decrease of the peak at 199 and 200 bp for the HEX-strand and 201 bp for FAM-strand. We can thus conclude that a 3′ exonuclease activity of proofreading DNA polymerase is involved in trimming the PCR amplicons.

4 Discussion

Microbial diversity studies are based on the amplification of marker genes using PCR. Owing to their higher DNA synthesizing fidelity and lack of +A-tail [20], proofreading DNA polymerases seem to be preferable for molecular fingerprinting [17]. However, using CE-SSCP, we have previously found that individual species produce multiple peaks [1]. These artifactual peaks distort biodiversity estimations in molecular profiling studies. Here, we found that this artifact is inherent to the proofreading DNA polymerase, and is due to an exonuclease activity that trims the 3′-end. We found this effect with all the proofreading enzymes studied here (Table 1, Figs. 1 and 2). Moreover, only 52% of the double-strand amplicons obtained with Isis were calculated to be blunt ended (Table 3), while the remaining fragments were asymmetric and heterogeneous (Table 2). To the best of our knowledge, this is the first study linking exonuclease activity to modifications in PCR amplicons.

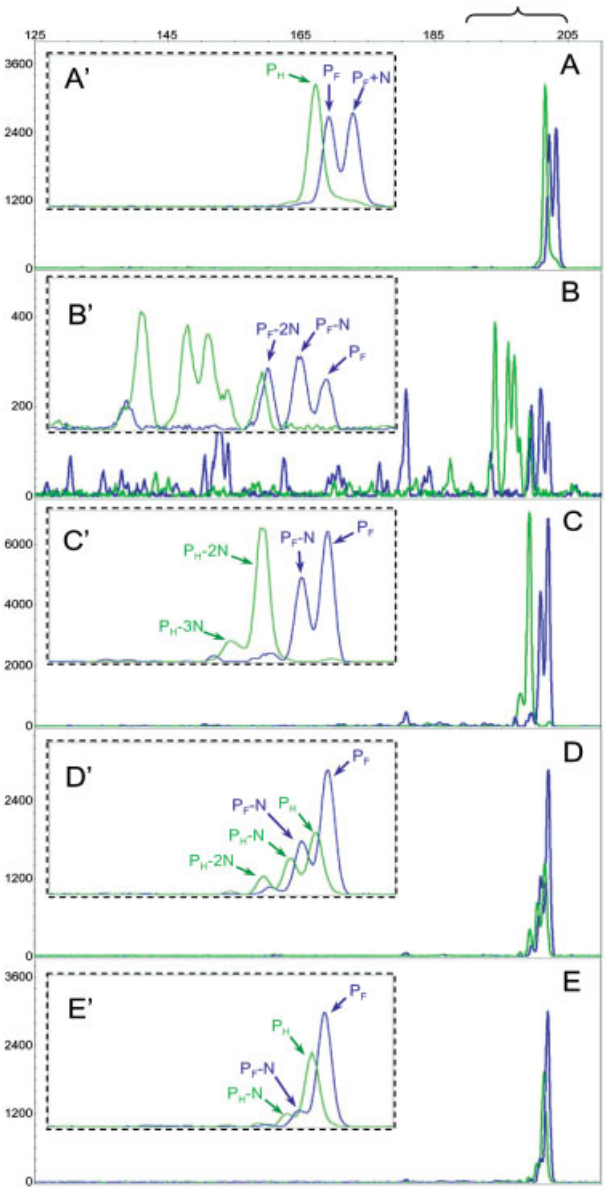

Figure 3. Trimming of the Ampli*Taq* Gold amplicons by Isis DNA polymerase. (A)–(E) Full-range electropherograms of CE-FLA profiles, (A′)–(E′) 190–205 region of electropherograms. (A) and (A′) no treatment, (B) and (B′) Isis treatment with 0 μM dNTP, (C and C′) treatment with 10 μM dNTP, (D) and (D′) treatment with 50 μM dNTP, (E) and (E′) treatment with 500 μM dNTP. Colors and peak attribution are as in Fig. 2.

4.1 The 3′–5′ exonuclease activity of proofreading DNA polymerases

This activity has already been described with thermosensible and thermostable DNA polymerases, when using short length (usually 17–20 mer) ss/dsDNA template or plasmid restricted fragments [19, 29, 30]. For instance, the Vent™ DNA polymerase (from *Thermococcus litoralis*) showed 3′–5′ exonuclease activity on dsDNA in the absence of dNTP [31]. The exonuclease activity was correlated with the polymerase activity [32], and is thought to be essential for proofreading [26, 33]. The double-strand exonuclease activity was attributed to "frayed" ends of dsDNA in the polymerase's active site [34, 35]. Actually, almost all the thermostable polymerases except the *Taq* polymerase possess this characteristic [19], with ssDNA as the preferred substrate. During PCR, trimming occurs in both strands, but is variably extensive, depending on many factors such as template, primer sequences, and concentration of dNTP (see below, data not shown).

4.2 Effect of dNTP concentration

The correlation between degradation and dNTP concentration was previously observed for the *Pfu* DNA polymerase, in a polymerase/exonuclease-coupled assay that used a radioactive primer [32]. At very low dNTP concentrations (0.01–1 μM) these primers were not elongated but degraded [32] whereas synthesis overcame degradation at 10 μM. In our study, we did not assay concentrations below 10 μM, but we did find that degradation clearly took place even at higher concentrations. Trimming of the 3′-end by Isis polymerase decreased as the concentration of dNTP increased, (Fig. 3), and was reduced to a few bases at 50 μM dNTP. With *Pfx*, for which the suggested dNTP concentration is 300 μM, the trimming also occurred in the absence of dNTP, but concentrations of 50 μM were not enough to complete the HEX-W49 strand (data not shown). At the concentration suggested by the manufacturer, the Platinum *Pfx* polymerase produced up to 85% of properly blunted PCR product, as compared to 52% produced by Isis (Table 3). At 500 μM, we did not observe any significant difference between CE-FLA peak patterns generated by Isis or *Pfx*. However, it did not seem possible to obtain 100% blunt-ended DNA even at 500 μM dNTP (Figs. 3E and E′). Moreover, the T4 DNA polymerase produced an N-1 peak, even when starting from +A-tailed PCR fragment

Table 3. Rates of blunted PCR products obtained with different DNA polymerase before (−T4) and after T4 DNA polymerase treatment (+T4) in denaturing CE analysis

DNA polymerase	Percentage[a)] of blunted PCR product	
	−T4	+T4
Ampli*Taq* Gold	48.73	73.33
Isis	52.41	72.61
Phusion	67	76.5
ProofStart	63.8	67.6
Platinum *Pfx*	85.2	77

a) Percentage obtained by multiplication of percentage of blunt form of each strand (Table 2).

(Fig. 2B). Exonuclease activity thus seems to remain in balance with polymerase activities but the former may be minimized using higher dNTP concentrations.

4.3 Consequences for molecular fingerprinting, SNP, diversity determination, and cloning blunt end PCR products

Proofreading DNA polymerases are widely used for the study of molecular profiles of microbial communities, probably because it is expected that their proofreading activity will reduce the number of "artificial" phylotypes. On the other hand, their characteristic shorter peaks introduce artificial phylotypes. In previous work, when degraded products were detected, the trimming activity was either attributed to contaminant nucleases or the electropherograms were simply misinterpreted [29, 32, 36]. Since these artifacts are inherent to proofreading DNA polymerases, it may be impossible to avoid them. In any case, neither increased concentrations of dNTP concentration, nor the use of primers with "clamped" 5′-end (GG, PIGtail, data not shown) [37, 38] succeeded in eliminating the shorter peaks. Although it can be hypothesized that the use of mixes of *Taq* and commercially available proofreading polymerases may render blunt products, the SSCP profiles obtained in our laboratory with these mixes were very similar to those obtained with DNA proofreading alone [1].

It is not desirable to add steps, such as reparation with T4 DNA, to high-throughput procedures. It seems that the most reasonable solution is to increase dNTP concentrations. There is, however, an inverse correlation between fidelity and dNTP concentration. This affects most of, if not all, proofreading DNA polymerases [20–22, 31, 32, 39] described as "next-nucleotide effect" [40]. For the Isis proofreading DNA polymerase the lower error rate (0.66×10^{-6} mutations/nucleotide/duplication for 1.5 mM $MgSO_4$) was found to be at 40 μM dNTP [41]. At a similar concentration (50 μM), the PCR product exhibits recessed 3′-termini for both strands. On the other hand, increasing dNTP concentration increased the error rate by a factor of 5 (3.05×10^{-6}) at 200 μM dNTP [41]. The choice is therefore between reducing artificial errors from polymerase misreading or increasing incidents of shorter peaks. It is worth considering *Taq* polymerases even if their error rate is roughly eight times those of Isis [41] at the recommended dNTP concentration and become three times that of Isis at the optimum concentration of dNTP to minimize shorter peaks. Moreover, the +A artifact can be controlled either by primer design (PIGtail or GC clamp) or by PCR conditions, leading to a single peak *per* species [37, 38]. These possibilities are currently under investigation and seem promising (Gury *et al.* in preparation) Proofreading DNA polymerases, with their low error rates, are well-suited to environmental analyses when using newer methods of high-throughput sequencing such as 454™ technology (Roche) or Solid™ system (Applied Biosystem).

In conclusion, Isis and other proofreading DNA polymerases generate PCR fragments with different proportions of 3′-recessed termini (Table 3). The recessed fragments are a consequence of the 3′–5′ exonuclease activity inherent to DNA polymerases that may be minimized but not eliminated. Future analysis of microbial diversity should take into account this artifact. Nevertheless, Platinum *Pfx* is a tested DNA polymerase that generates few artifacts and displays the best product yield of blunted PCR amplicon (up to 85%), but, because of its relatively high cost, its use is not appropriate for high-throughput environmental studies. For molecular profiling of microbial communities by FLA or SSCP, we recommend the use of *Taq* DNA polymerase, with appropriate optimization of primers and PCR conditions as necessary.

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Annexe B

Snow cover dynamics and phylogenetic structure of bacterial communities in alpine tundra soils

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Running title: Bacterial communities of alpine soils

ABSTRACT

Because of their role in nutrient cycling, soil bacterial communities play an essential role in the response of tundra ecosystems to global warming. The variation in snow cover dynamics in alpine tundra has a profound impact on ecosystem structure and functioning, but its relation with the dynamics of bacterial communities remains unknown. Here we compared the yearly course of phylogenetic structure of soil bacterial communities sampled from early snowmelt locations (ESM) and late snowmelt locations (LSM) in temperate alpine tundra. The analysis of 1700 *ssu* sequences from both locations and 4 seasons revealed that the overall phylogenetic structure of bacterial communities was strongly associated with temporal change in snow cover. Temporal variations were mainly due to strong environmental filtering, as indicated by the phylogenetic clustering of *Acidobacteria, Actinobacteria* and *α-proteobacteria* at the end of winter in ESM and of *β-,γ-proteobacteria* and *CFB* at both locations at autumn. The drop of soil pH in ESM after soil thawing possibly induces selection of *Acidobacteria SD1*. Autumn litter-fall was associated with the selection of *Pseudomonadales*, *Burkholderiales*, *Flavobacteriales* and *Sphingobacteriales*. Our results show that bacterial communities are highly influenced by seasonal environmental variation, indicating that they will respond strongly to changes in climatic drivers.

1. INTRODUCTION

The mechanisms driving the assembly of microbial communities are still largely unexplored, despite the central role they play in ecosystem functioning and response to global changes. In particular, alpine ecosystems, which are characterised by abrupt environmental gradients (Körner, 1999; Litaor et al., 2002; Edwards et al., 2007), are predicted to be heavily impacted by climate changes in future decades. Indeed, strong modifications in the dynamics of snow cover and length of the growing season are expected (Beniston, 2005), and these changes could have strong effects on plant and microbial communities (Theurillat and Guisan, 2001; Choler, 2005; Bryant et al., 2008). At the two extreme of the snow cover gradient, alpine tundra from Early and Late Snow Melt locations (hereafter ESM and LSM), though spatially very close, exhibit contrasting plant cover, soil characteristics (including soil carbon stock), and microbial communities (Litaor et al., 2002; Choler, 2005; Edwards et al., 2007; Zinger et al., 2009). Therefore, this gradient offers a unique opportunity to examine how the natural dynamics of snow cover may impact soil microbial communities and soil functioning. This is of particular relevance in the light of the measured and predicted changes of snow regimes in arctic and alpine tundra (IPCC, 2007).

In recent years, phylogenetic information has been increasingly used to infer the ecological and evolutionary mechanisms driving contemporary community assembly (Webb et al., 2002; Vamosi et al., 2009). This approach is based on the rationale that phylogenetic distance between two species or taxa is a measure of ecological similarity, assuming that ecological characteristics of taxa are phylogenetically conserved. Thus, if one assumes that habitat filtering is the major mechanism of community assembly, it is expected that local environmental conditions will only allow the occurrence of related species (or phylotypes) that share specific ecological characteristics, leading to phylogenetic clustering of communities (Webb et al., 2002; Cavender-Bares et al., 2006). However, when resource competition is the dominant mechanism, closely related taxa should tend to mutually exclude each other within local communities, leading to a pattern of phylogenetic overdispersion (Webb et al., 2002; Cavender-Bares et al., 2006).

So far, the assembly rules of microbial communities have been little investigated (but see (Horner-Devine and Bohannan, 2006; Newton et al., 2007; Bryant et al., 2008)). Such studies have usually revealed a significant phylogenetic clustering, suggesting that drastic selective pressures alter the structure of microbial communities. However, more data are needed to confirm whether phylogenetic clustering is common in microbial communities. Moreover, the study of variation in microbial phylogenetic structure over time and space may provide new insights into the spatial distribution and succession of these communities and how environmental changes may influence their activity.

Here, we studied bacterial communities over the course of one year in ESM and LSM locations. A previous study highlighted the differences between these two communities and their seasonal variations (Zinger *et al.*, 2009). However, the temporal and spatial variations in the bacterial phylogenetic structure in these two communities are poorly understood.

First, analysis using Ribosomal Date Project (RDPII) tools confirmed the previous structure of the subphylum level. Second, we used two different statistical approaches to assess phylogenetic structure: one based on the computation of the beta (between-locations and between-date) component of phylogenetic diversity (from species abundance data), and one based on the computation of indices depicting the phylogenetic structure of each local community at one given date (from patterns of species presence/absence).

2. MATERIAL AND METHODS

Study site, sample collection and bacterial ssu sequences: The study area is located in the Grand Galibier massif (southwestern Alps, France, 45°05' N, 06°37' E). The sampling site displays a topographical gradient comprising two neighbouring habitats: ESM and LSM, located in an area of less than 100 m^2. In harsh winter conditions, snow-cover in LSM plays a protective role for bacteria communities by keeping the soil temperature around 0°C. Conversely, ESM soils are submitted to freeze-thaw cycles because of intermittent snow coverage. These differences lead to a longer growing season in ESM and result in differences in plant cover and soil characteristics between locations (Choler, 2005). Slow-growing, stress-tolerant plants dominate ESM (*Kobresia myosuroides, Dryas octopetala, Carex curvula All. subsp. rosae*), while LSM is dominated by fast-growing species (*Carex foetida, Salix herbacea L., Alopecurus alpinus Vill., Alchemilla pentaphyllea L.*). Five soil samples from each location were collected in 2005 on June 24th (spring), August 10th (beginning of growing season) and October 10th (after litter-fall), and in 2006 on May 3rd (late winter). During the late-winter sampling, the mean soil temperature of ESM rose above 0°C for 10 days, and the mean for LSM was around 0°C. The LSM soil was covered by >2.5 m of snow pack and was waterlogged. The soil samples were immediately transported to the laboratory in sterile conditions and sieved at 2 mm before DNA extraction. The clone libraries' construction has been previously described (Zinger *et al.*, 2009). The accession numbers of the *ssu* sequences analysed in the present study are FJ568339 to FJ570564.

Sequence data treatment: The chimerical sequences were identified using the Bellerophon program (Huber *et al.*, 2007) and removed from the dataset. The length of the remaining sequences (1077 for LSM and 1311 for ESM, 270 and 320 sequences per location/date) ranged from 400 to 900 bp. The taxonomic assignment of *ssu* sequences was done using the Classifier (Cole *et al.*, 2003) tool from the Ribosomal Database Project (RDPII). The closest matching sequences, identified with Match (Cole *et al.*, 2005), were used as references for the phylogenetic analysis. The multiple alignments were performed with 1707 sequences using the ClustalW algorithm (Thompson *et al.*, 1994) for each phylum. For *Proteobacteria*, *α-proteobacteria* were analysed separately from other *proteobacteria* because of their phylogenetic distance (Kersters *et al.*, 2006).The phylogenetic analysis was performed with MEGA 3.1 (Kumar *et al.*, 2004) by using the distance of Kimura2 to calculate evolutionary distances and Neighbour-Joining to construct phylogenetic dendrograms. The robustness of the phylogenetic tree was evaluated by performing 1000 bootstraps.

Statistical Analysis: The Classifier tool from RDPII allowed the assessment of phylotypes' abundances at different taxonomic levels. From the phylotypes' abundances at the phylum and sub-phylum levels, we analysed seasonal and spatial differences for each of the bacterial taxa with the Pearson's Chi-squared test and Library Compare (Cole *et al.*, 2005) from RDPII. We also computed a frequency-based distance matrix using Edward's distance and constructed Neighbour-Joining dendrograms, the robustness of which was assessed by performing 1000 bootstraps. The latter data treatment was performed using R software (R Development-Core Team, 2008).

To study bacterial phylogenetic structure, we used two different approaches. First, we used the framework provided by Rao's quadratic entropy, which allows the partitioning of phylogenetic diversity into its alpha and beta components (Hardy and Senterre, 2007). We computed the Qst index (β phylogenetic diversity), which measures how communities are structured, taking into account taxa phylogenetic relationships and their local abundances, following (Villeger and Mouillot, 2008). We computed Qst between the ESM and LSM sites, between sampling dates, and between dates and sites, in order to estimate the spatial, temporal and spatio-temporal variation, respectively, in communities' phylogenetic structure. Significance of Qst estimates was assessed by comparing the estimated value to the values obtained by drawing 9,999 permutations of phylotypes across the phylogenetic tree.

Second, we computed for each local community the nearest taxon index (hereafter NTI), which quantifies the degree of phylogenetic clustering of taxa in local communities given their patterns of presence/absence in communities and their phylogenetic relationships. The NTI of each community was defined as [–(MNND – MNNDnull)/ SD(MNNDnull)], where MNND is the mean phylogenetic distance of species to their nearest neighbour in the phylogeny (Webb *et al.*, 2002). MNNDnull is the mean MNND for the same communities after permuting taxa across the phylogenetic tree 9,999 times, and SD(MNNDnull) is the standard deviation of these permuted MNND values. As defined here, a positive NTI indicates phylogenetic clustering within a given community, whereas a negative value indicates phylogenetic overdispersion in the local community (R Development-Core Team, 2008). We chose to use only the NTI index, and not both the NTI and NTR (net relatedness index), as done elsewhere (Webb *et al.*, 2002), because phylogenetic trees were only phenetic.

3. RESULTS

Comparison of bacterial communities using taxonomic information

We have obtained 8 *ssu* libraries, 1 per date and site. The taxonomic affiliation of phylotypes present in ESM and LSM libraries was assessed using Classifier from RDPII. The spatial variations of the main phyla are shown in Table 1. Briefly, the most striking differences between ESM and LSM were found in *Acido-* and *Actinobacteria*. The *Proteobacteria* group (*α-*, *β-*, and *δ-Proteobacteria*) was also found to vary between the two locations. These results confirm that the spatial distributions of bacterial groups in LSM and ESM are different at the phylum and sub-phylum levels (data not shown).

Table 1: Principal groups of bacteria identified by RDPII, relative abundance in ESM and LSM and Library Compare and Chi-Squared tests of significance

Phylum bacteria	% in LSM	% in ESM	P by RDPII	P by chi-square
Acidobacteria	42 ± 3	22± 11	6E - 14	8.75E - 07
Actinobacteria	6± 4	18 ± 3	6E - 14	2.744E - 06
α-Proteobacteria	12 ± 4	19 ± 4	0.00006	0.026
β-Proteobacteria	3.5 ± 0.7	7 ± 3.8	0.0003	0.021
γ-Proteobacteria	5.8 ± 3.7	6 ± 2.07	0.9	0.76
DeltaProteobacteria	1.5 ± 1.3	4 ± 2	0.001	0.03
CFB	5.6 ± 3	8.3 ± 3.5	0.008	0.154
Firmicutes	1 ± 0.4	0.9 ± 0.5	0.99	0.8
Gemmatimonadetes	2.4 ± 1.3	3 ± 0.1	0.6	0.5
Other	0.8 ± 1	1 ± 1.07	0.4	0.5
Unclassified bacteria	18.9 ± 4	10 ± 2	NA	0.001

In order to validate the seasonal and spatial variations at the phylum and sub-phylum levels, we constructed a Neighbour-Joining tree based on their relative abundance frequencies (Fig. 1). There was a marked effect of location in the clustering of bacterial phyla, except in May (Fig. 1A). At the subphylum level, the largest distance between ESM and LSM was observed in June and August and decreased from October to May (Fig. 1B).

Comparison of bacterial communities using phylogenetic information

In order to gain insights into the effect of location and seasonal variation on the phylogenetic structure of bacterial communities, we constructed phylogenetic trees for each phylum (see Suppl. Fig. 1, 2, 3, 4 and 5).

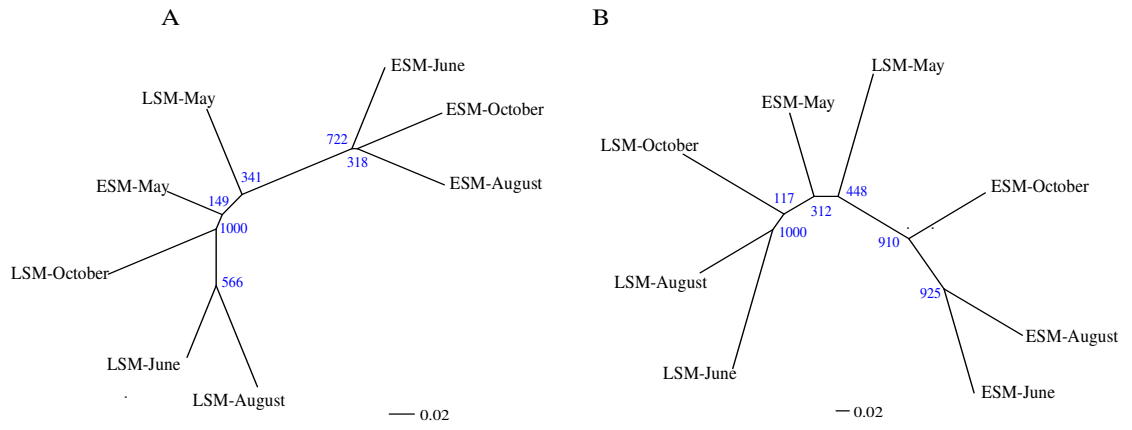

Figure 1: Spatial and seasonal variation of bacterial community**.** The dendrograms are based on phylotype frequencies at the phylum **(A)** and sub-phylum levels **(B).** The bootstrap value is shown for each node.

The phylogenetic β diversity was computed using the local relative abundance of each phylotype to determine how locations, seasons and the interaction between locations and seasons contribute to the structure and assembly of bacterial communities. As shown in Fig. 2, the estimators of spatial, temporal and spatio-temporal β diversities were always significantly different from the value computed under a null model of phylotype distribution. Phylogenetic β diversity was always found to be much higher between sampling dates than between sampling sites. Spatial and temporal environmental variations seemed to exert a synergistic effect on bacterial community structure, with spatio-temporal variation in phylogenetic composition always being higher than both spatial and temporal variation taken alone.

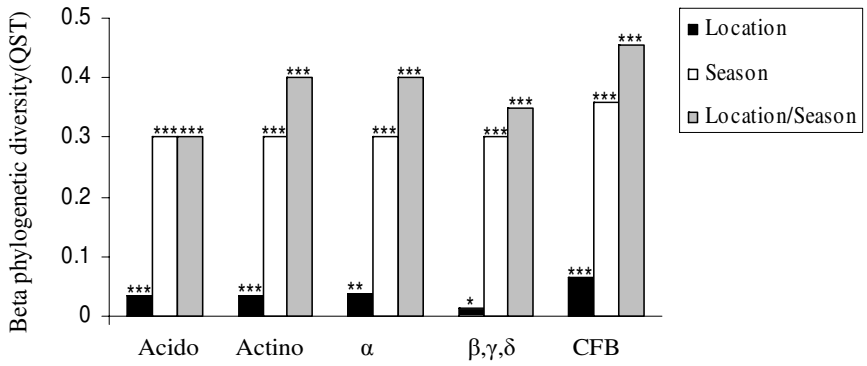

Figure 2: β phylogenetic diversity between ESM and LSM locations. Significance of Qst estimates was assessed by comparison to a null model obtained with 9999 permutations and is indicated as follows: ***: P<0.001, **: P< 0.01, *: P<0.05.

In the ESM location, bacterial communities showed no significant pattern of phylogenetic clustering during the growing season until October, when *CFB* and *β- γ-δ Proteobacteria* were phylogenetically clustered. In May, *Acidobacteria, Actinobacteria,* and *α-Proteobacteria* communities also began to display significant clustering (Fig. 3A). Interestingly, bacterial communities in the LSM location also displayed phylogenetic clustering of *β-/γ-/δ- Proteobacteria, CFB*, *Acidobacteria* and *Actinobacteria* in October. Also, the *Acidobacteria* community showed a significant phylogenetic clustering all year round (Fig. 3B).

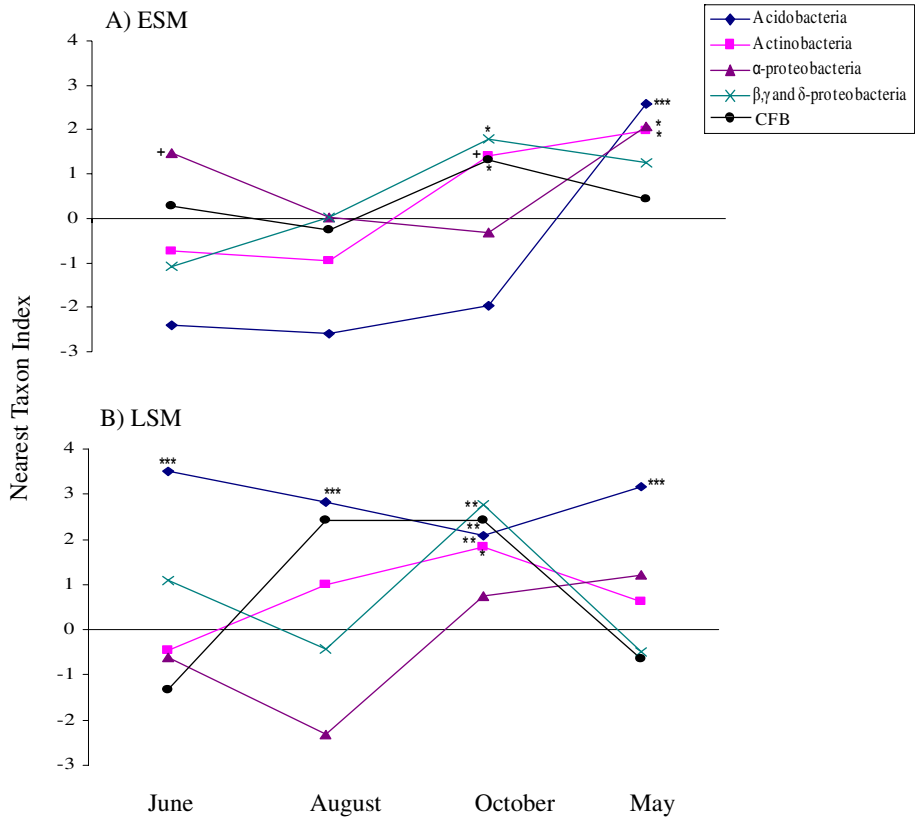

Figure 3: Seasonal variation in the phylogenetic structure of bacterial communities. A: ESM and B: LSM. NTI positive index values present phylogenetic clustering, and negative values indicate phylogenetic overdispersion. The significance of NTI estimates is shown as follows: ***: P<0.001, **: P< 0.01, *: P<0.05, +: P<0.1

Distribution of bacterial groups at finer taxonomic scale

We characterised the distribution of the main bacterial orders in each sample (Fig. 4). In ESM and LSM, the *Acidobacteria* were mainly represented by subdivisions (SD) 1 to 7 and 17. The first striking feature is the spatio-temporal variation of *SD1*, which largely dominated

the LSM libraries and, in May and October, the ESM library (Fig. 4A). As shown in Fig. 4B, *Actinobacteria* were represented by ten suborders and was more abundant in ESM in June and August. Interestingly, *Streptosporanginaceae* became more abundant in both locations in October and May. *α-Proteobacteria* were found to be more abundant in ESM, except in May, and were mostly represented by *Rhizobiales* (Fig. 4C).

The *β-Proteobacteria* were more abundant in ESM than LSM due to an increase of the order *Burkholderiales* in October (Fig. 4D). The *γ-Proteobacteria* did not display a significant difference between the two locations but increased in the October libraries of both locations, mainly due to phylotypes affiliated with *Pseudomonadales* (Fig. 4E). The clustering of these classes during October in both locations (Fig. 3) may correspond to the increasing of *Pseudomonadales* and *Burkholderiales*. The CFB phylum was more frequent in October in both locations, exemplified by *Flavobacteriales* and *Sphingobacteriales* (Fig. 4F).

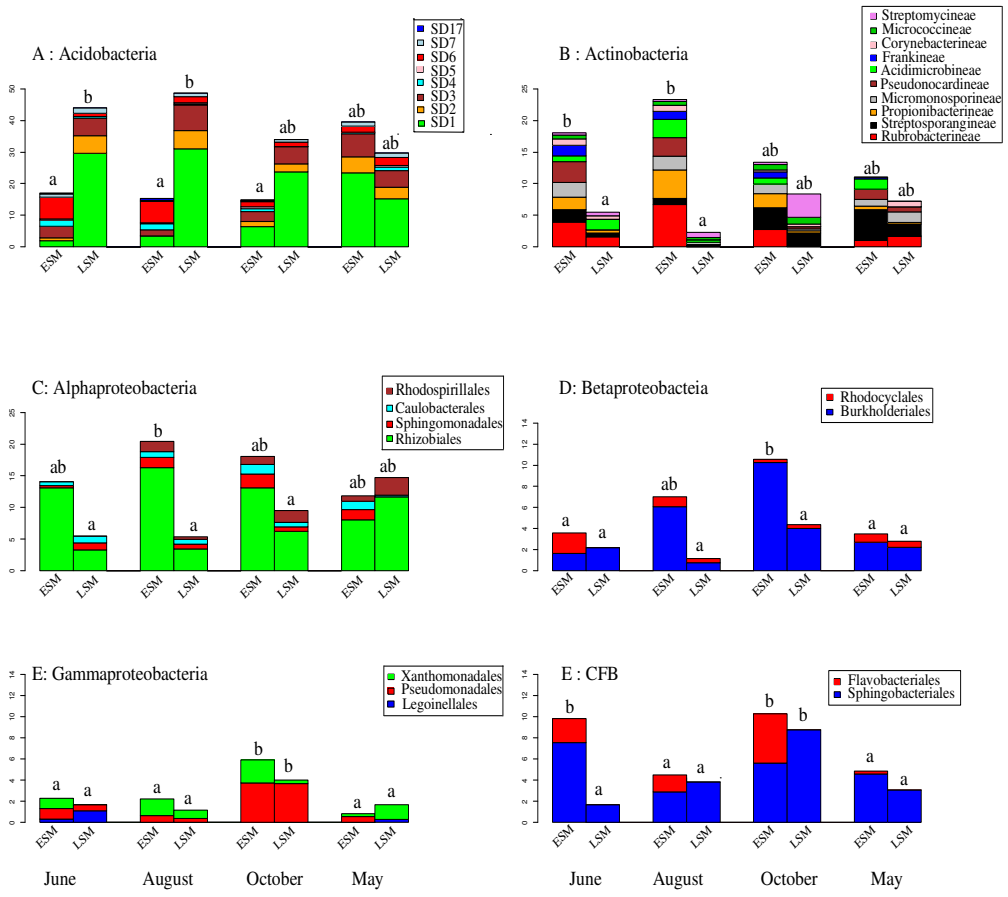

Figure 3: Relative abundance of bacterial phyla and class. A): *Acidobacteria*, B): *Actinobacteria*, C): *α-Proteobacteria,* D): *β-Proteobacteria*, E): *γ-Proteobacteria* and F): *CFB*. Significant differences between the two studied locations and seasons are indicated by small letters.

4. DISCUSSION

The community assembly of bacteria, in particular in alpine tundra, is poorly understood. Previous studies using PLFA have reported similar patterns in alpine tundra habitats (Bjork *et al.*, 2008). However, due to the previously used methodology, the bacterial clades responsible for these seasonal changes remain poorly characterised. The few molecular studies regarding alpine tundra showed seasonal variations in a *K. myosuroides* alpine meadow (Lipson and Schmidt, 2004; Lipson, 2007). In this study we examined the phylogenetic structure of bacterial communities from alpine tundra soils sampled in Early and Late Snow Melting locations, representing the two extremes of an environmental gradient.

Our sampling was timed by the natural snow cover dynamics observed in these contrasting locations. Our results showed a significant effect of location (ESM *vs.* LSM) at the phylum and subphylum levels (Fig. 1), and even at the finer taxonomic level (Fig. 2). Thus, the effect of location on β diversity seems to be related to the plant-growing season. Plants can influence soil bacteria communities via root exudates (Bais *et al.*, 2006), and thus different plant cover may recruit different bacterial phylotypes (Grayston and Campbell, 1996; Maloney *et al.*, 1997). LSM and ESM display different plant species compositions, which can partially explain the observed differences in their bacterial communities. Actually, the correlation between plant species and soil bacterial communities has already been explored (Buyer 2002), even in snow-covered ecosystems (Yergeau et al., 2007b; Yergeau et al., 2007a).

However, there are few reports on the co-occurrence of specific bacterial phylotypes and plant species. One interesting finding was the presence of *Acidobacteria SD6* in ESM *(*dominated by *K. myosuroides*), which was nearly absent from LSM. Actually, this *Acidobacteria* subdivision was found to be associated with the rhizosphere (Ludwig *et al.*, 1997; Zhou *et al.*, 2003) and in soils covered by *K. myosuroides* (Lipson and Schmidt, 2004). Given the lack of cultivable species of this subdivision, environment studies are useful tools to pinpoint a putative interaction with specific plants. Moreover, ESM is expected to contain more recalcitrant litter than LSM (Choler, 2005), which may drastically reduce the availability of nitrogen for the plant (Baptist *et al.*, 2008), suggesting that this location relies on symbiotic N_2 fixation. This hypothesis is supported by the relative abundance of *Rhizobiales* (Fig 4), which are known for their ability to fix N_2 (Parker, 2007).

However, within bacterial clades, the analysis of phylogenetic β diversity showed that temporal variation in bacterial phylogenetic structure was greater than the variation between

locations (Fig. 2), suggesting that the temporal fluctuations of environmental conditions are a major determinant for the community assembly of bacteria in alpine tundra. Indeed, this was due to a strong phylogenetic clustering of bacterial communities that was pronounced in May for *Acidobacteria* in ESM sites and in October for *CFB*, *β-* and *γ-Proteobacteria* in both ESM and LSM sites. This strong phylogenetic clustering suggests the existence of drastic environmental filters in determining the function of alpine bacterial communities. As shown elsewhere, the environmental requirements of different bacterial phylotypes may show a phylogenetic signal, causing closely related phylotypes to have similar environmental requirements (Horner-Devine and Bohannan, 2006; Newton et al., 2007; Bryant et al., 2008) and therefore respond similarly to spatio-temporal variation in environmental variables (such as the ones imposed by snow cover dynamics).

The clustering in May occurred concomitantly with the low soil temperature and the drop of ESM's soil pH (see (Zinger *et al.*, 2008; Zinger *et al.*, 2009)), which was mainly due to the increase of *Acidobacteria SD1* in ESM. Indeed, *Acidobacteria SD1* was found to be more abundant in LSM (pH ~ 5.5) than in ESM (pH ~ 6.5) during the productive season (Fig 4A). Interestingly, the decrease of pH in late winter in ESM was concomitant with the increase of *SD1*. *Acidobacteria* were previously found to be more abundant at pH lower than 5 (Lauber *et al.*, 2008), while *Acidobacteria SD1* have been reported to have a preference for acidic pH (Sait *et al.*, 2006). Thus, in a larger context than alpine soils, it is conceivable that *Acidobacteria* abundance in soils with pH<5 is due mainly to *SD1*. A larger molecular survey is needed to support this hypothesis. The phylogenetic clustering of community *Actinobacteria* in late winter concords with observations in aquatic ecosystems (Newton *et al.*, 2007). Hence, the pH appears to be a determinant filter of bacterial communities, confirming the observations of Fierer (Fierer and Jackson, 2006) and Lauber (Lauber *et al.*, 2008).

The clustering in October occurred after plant senescence and was concomitant with the increase of *Pseudomonadales*, *Burkholderiales*, *Flavobacteriales* and *Sphingobacteriales* (Figs. 4D, E, F). These orders comprise species that are heterotrophic and are associated with the degradation of complex organic matter and/or are plant or human pathogens (Halden et al., 1999; Cottrell and Kirchman, 2000; Sheng and Gong, 2006; Rein et al., 2007; Manasiev et al., 2008). Since this sample was taken after plant senescence, the bacterial communities thriving on root exudation were probably diminished. Given the ecological characteristics of the bacterial phylotypes and the presence of plant litter, we hypothesise that the observed

patterns resulted from a change in resource input, namely a shift from root exudates to plant litter.

CONCLUSION

In alpine tundra, the strong mesotopographical gradients trigger important differences in the composition and diversity of plant and microbial communities. Here we have shown that the soil bacterial communities at the two extremes of a snow cover/mesotopographical gradient display different phylogenetic structures and phylotype abundance. The phylum and subphylum levels of bacterial communities highlighted the effect of snow cover regimes, which was confirmed by β diversity analysis. The seasonal effect strongly impacted bacterial phylogenetic structure. This study found that the bacterial community assembly is strongly dependent on environmental conditions and that changes in nutrient resources coupled with soil temperature and soil pH may constitute strong filters on bacterial communities. However, further research is needed to assess the role of each environmental factor in the assembly of bacterial communities in alpine tundra. Finally, this paper also calls for more attention to the impact of global change in alpine tundra, which may deeply impact soil bacterial communities and thus ecosystem processes.

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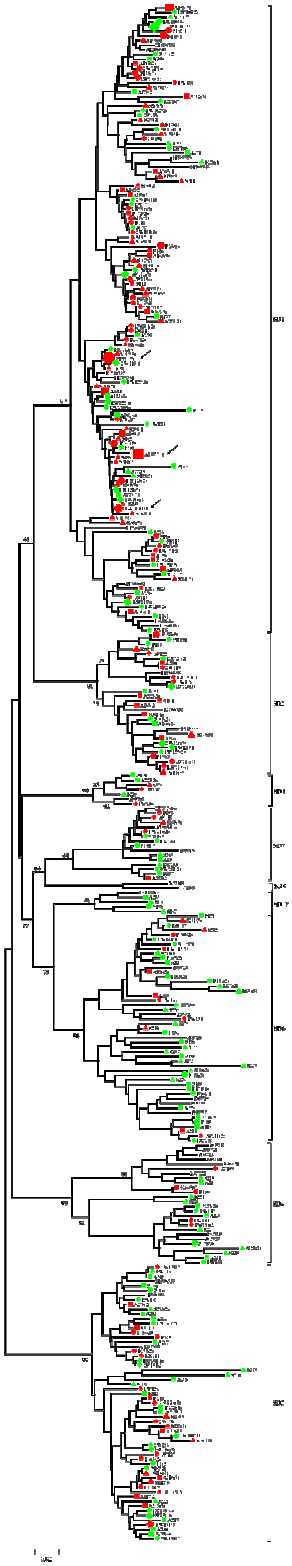
SUPPLEMENTARY MATERIAL

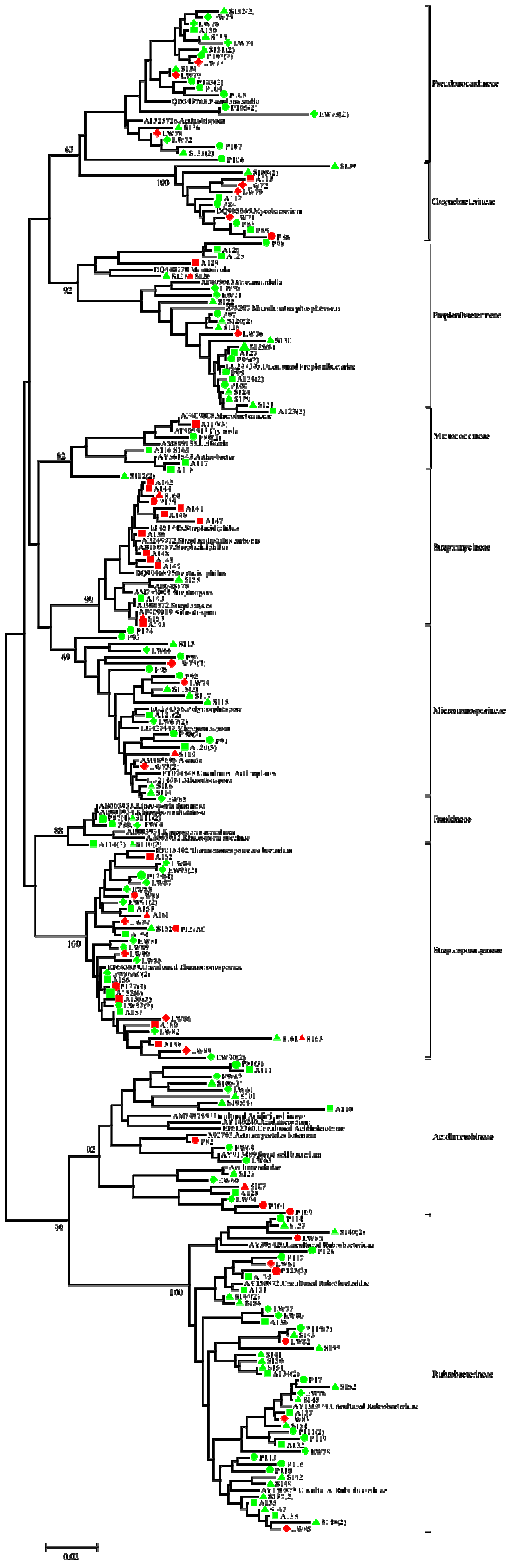
Supplementary Figures shows the NJ phylogenetic tree of *Acidobacteria* (SF 1), *Actinobacteria* (SF2), *α-proteobacteria* (SF3), *β-γ-δ-proteobacteria* and CFB (SF 4) and CFB (SF5).

Spring clones begin with P, summer clones with S, autumn clones with A and late winter clones with EW and LW. ESM and LSM identified green and red, respectively. The different season are showed by symbols: Circle: June, Triangle: August, square: October and diamond: May

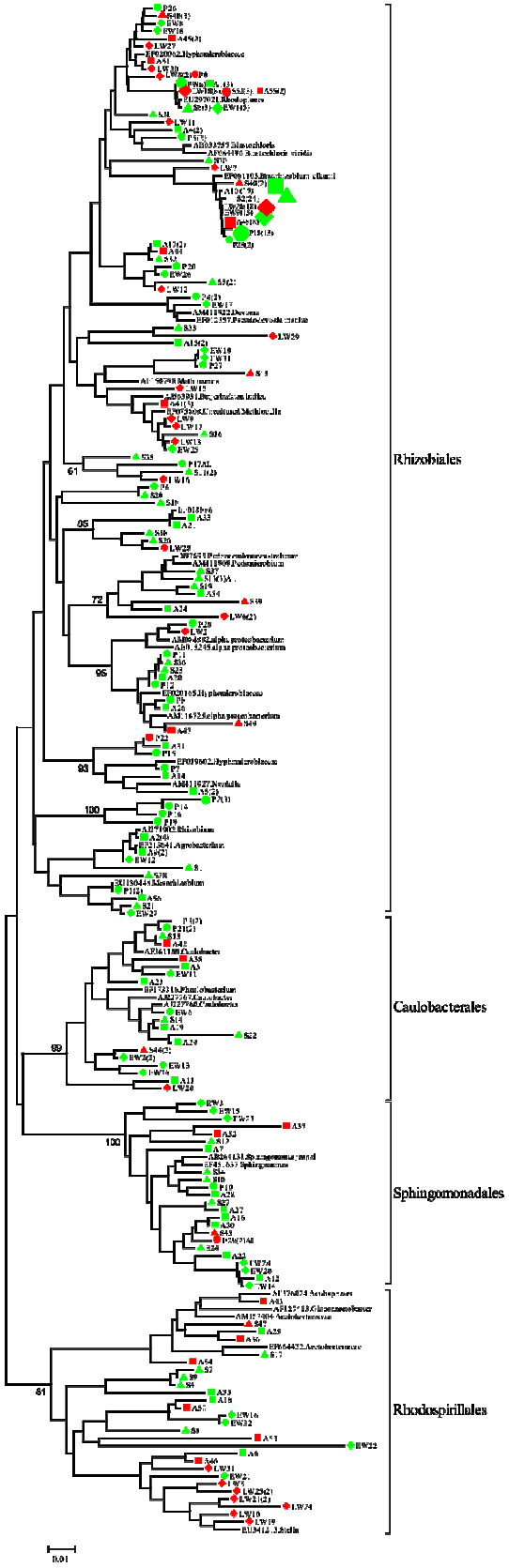
Supplementary Figure 1



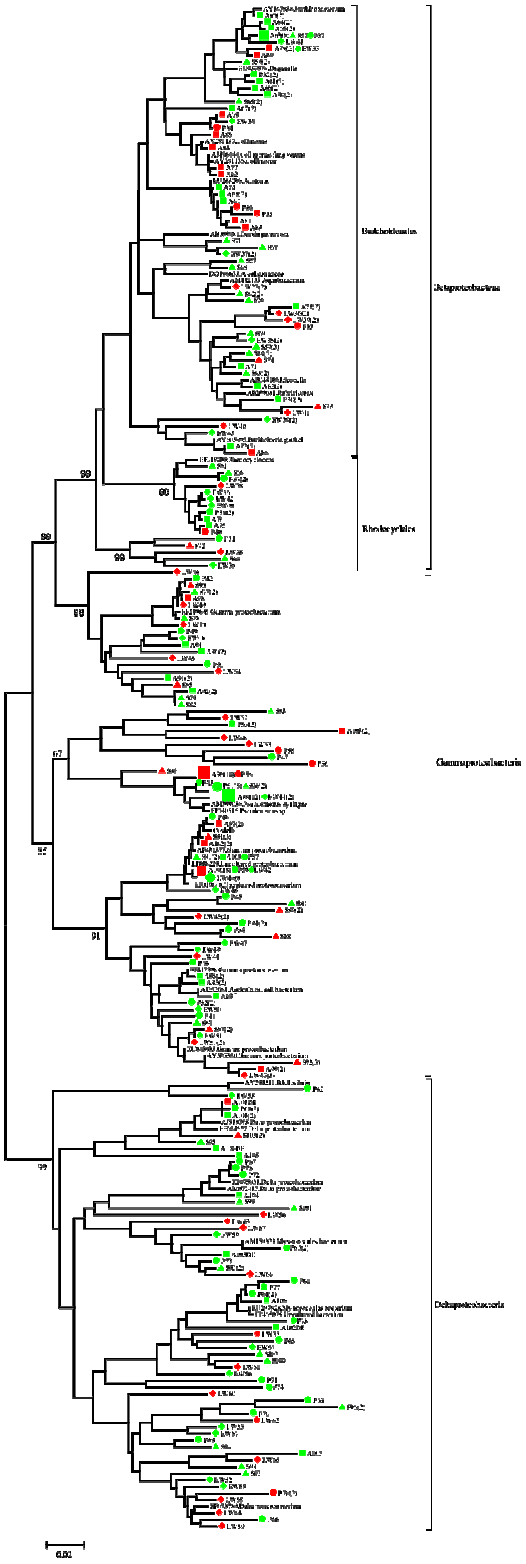
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

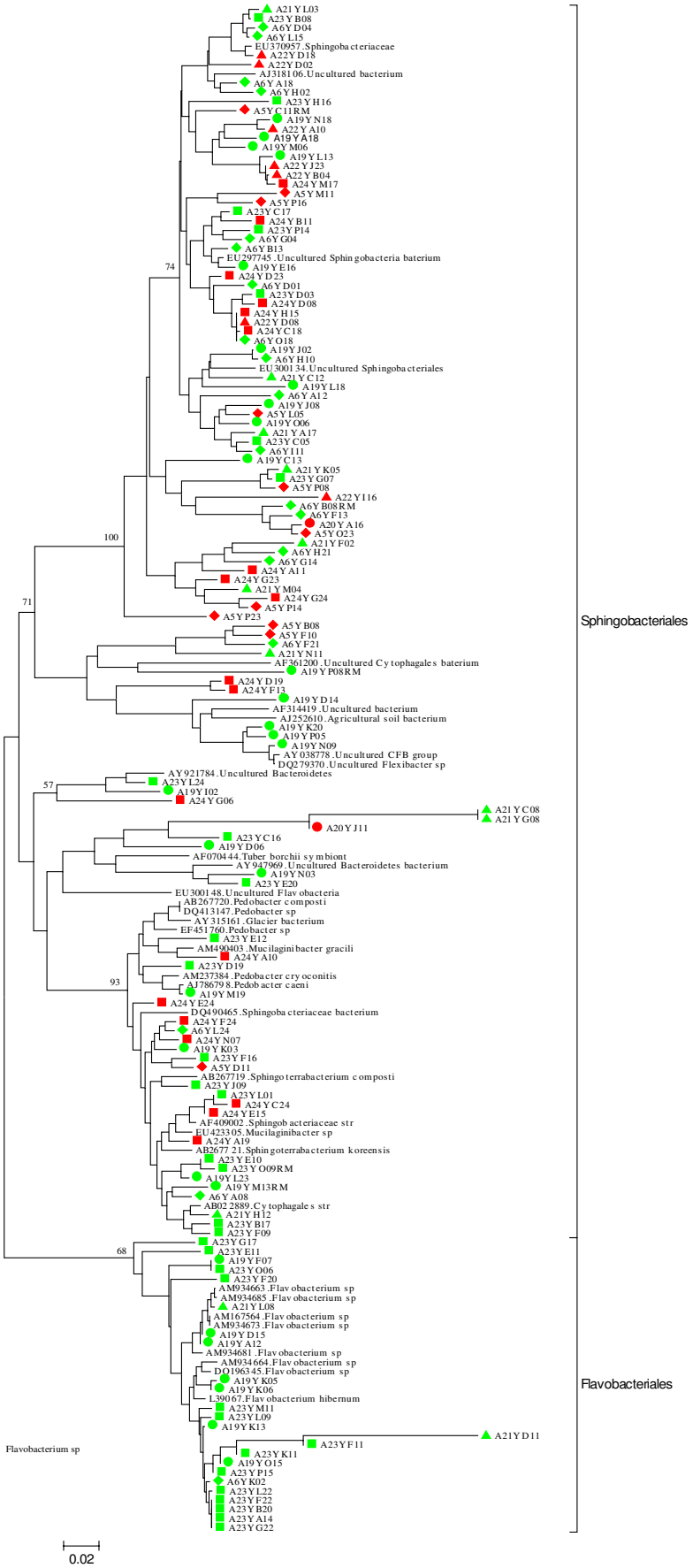


Table S3: Correlation between codes used in tree phylogenetic and corresponding accession number.

A:Acidobacteria

Sample	Code	Accession number	Sample	Code	Accession number	Sample	Code	Accession number
A19YI18RM	P130	FJ568495	A24YM23RM	A196	FJ569787	A22YB10RM	S184	FJ569094
A19YL05RM	P131	FJ568541	A24YO06RM	A197	FJ569808	A22YC01RM	S186	FJ569103
A20YA02RM	P132	FJ568632	A5YB03RM	LW101	FJ569854	A22YC04RM	S187	FJ569105
A20YA15RM	P133	FJ568637	A5YB21RM	LW102	FJ569870	A22YC16RM	S189	FJ569114
A20YB10RM	P134	FJ568648	A5YD10RM	LW103	FJ569906	A22YD12RM	S190	FJ569128
A20YB17RM	P135	FJ568652	A5YE12RM	LW104	FJ569932	A22YD15RM	S191	FJ569131
A20YC18RM	P136	FJ568666	A5YG20RM	LW105	FJ569986	A22YE04RM	S192	FJ569141
A20YD06RM	P137	FJ568674	A5YG24RM	LW106	FJ569990	A22YE16RM	S194	FJ569147
A20YD14RM	P138	FJ568679	A5YI03RM	LW107	FJ570016	A22YE20RM	S195	FJ569148
A20YE19RM	P139	FJ568692	A5YI14RM	LW108	FJ570024	A22YE22RM	S196	FJ569149
A20YF06RM	P140	FJ568698	A5YJ14RM	LW109	FJ570047	A22YF07RM	S197	FJ569155
A20YF16RM	P141	FJ568701	A5YJ20RM	LW110	FJ570053	A22YF16RM	S198	FJ569160
A20YG05RM	P142	FJ568712	A5YJ21RM	LW111	FJ570054	A22YG08RM	S199	FJ569170
A20YH06RM	P143	FJ568716	A5YL22RM	LW112	FJ570099	A22YH12RM	S200	FJ569181
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A20YK22RM	P145	FJ568739	A5YN11RM	LW114	FJ570134	A22YK03RM	S202	FJ569215
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A20YN013RM	P147	FJ568763	A5YO01RM	LW116	FJ570148	A22YL03RM	S204	FJ569227
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A21YD09RM	S171	FJ568843	A6YB09RM	EW103	FJ570224	A20YF21RM	P151	FJ568705
A21YE23RM	S172	FJ568874	A6YC22RM	EW104	FJ570258	A20YG01RM	P152	FJ568708
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Sample	Code	Accession number	Sample	Code	Accession number	Sample	Code	Accession number
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A24YJ06RM	A217	FJ569732	A19YF02RM	P174	FJ568428	A22YP01RM	P184	FJ569277
A24YM12RM	A218	FJ569781	A19YH13RM	P175	FJ568475	A19YK17RM	P185	FJ568531
A24YP15RM	A219	FJ569828	A19YI15RM	P176	FJ568492	A21YA05RM	S246	FJ568782
A5YB24RM	LW124	FJ569873	A19YJ01RM	P177	FJ568500	A21YO01RM	S247	FJ569033
A5YC01RM	LW125	FJ569874	A19YO22RM	S228	FJ568610	A23YK15RM	A233	FJ569494
A5YC24RM	LW126	FJ569896	A19YP06RM	P178	FJ568616	A19YA19RM	P186	FJ568353
A5YF07RM	LW128	FJ569950	A19YP23RM	P179	FJ568629	A19YL08RM	P187	FJ568543
A5YI06RM	LW129	FJ570019	A21YA01RM	S229	FJ568779	A19YP07RM	P188	FJ568617
A5YK04RM	LW130	FJ570061	A21YA04RM	S230	FJ568781			
A5YM01RM	LW131	FJ570101	A21YB18RM	S231	FJ568809			
A5YM06RM	LW132	FJ570106	A21YC05RM	S232	FJ568840			
A5YO06RM	LW133	FJ570153	A21YE16RM	S233	FJ568868			
A6YD14RM	EW132	FJ570274	A21YF20RM	S234	FJ568891			
A6YE24RM	EW133	FJ570308	A21YG07RM	S235	FJ568899			
A6YF02RM	EW134	FJ570310	A21YH04RM	S237	FJ568917			
A6YF11RM	EW135	FJ570319	A21YH19RM	S238	FJ568926			
A6YH07RM	EW136	FJ570362	A21YI08RM	S239	FJ568935			
A6YI02RM	EW137	FJ570380	A21YO18RM	A224	FJ569044			

A6YM17RM	EW138	FJ570489	A23YC24RM	A225	FJ569345			
A6YO10RM	EW139	FJ570527	A23YF24RM	A226	FJ569404			
A6YP08RM	EW140	FJ570549	A23YG04RM	A227	FJ569408			
A6YP15RM	EW141	FJ570555	A23YG18RM	A228	FJ569419			
A22YA14RM	S217	FJ569078	A24YA02RM	A229	FJ569583			
A22YB12RM	S218	FJ569095	A24YK19RM	A230	FJ569756			
A22YE12RM	S219	FJ569144	A24YL03RM	LW140	FJ569763			
A19YD05RM	P165	FJ568391	A5YI04RM	LW141	FJ570017			
A19YJ11RM	P166	FJ568507	A5YL09RM	LW142	FJ570088			

B: Actinobacteria

Sample	Code	Accession number	Sample	Code	Accession number	Sample	Code	Accession number
A19YO18RM	P81	FJ568608	A21YE12RM	S125	FJ568865	A23YA17RM	A132	FJ569297
A20YO05RM	P101	FJ568766	A21YE20RM	S126	FJ568871	A23YC08RM	A134	FJ569333
A20YL01RM	P109	FJ568742	A21YM11RM	S127	FJ569003	A23YC23RM	A135	FJ569344
A21YB08RM	S100	FJ568801	A21YO14RM	S129	FJ569041	A23YD11RM	A136	FJ569353
A21YP07RM	S105	FJ569055	A21YO16RM	S130	FJ569043	A23YE23RM	A137	FJ569384
A22YP07RM	S107	FJ569279	A23YB10RM	A123	FJ569313	A23YK01RM	A138	FJ569482
A23YL11RM	A110	FJ569510	A23YH04RM	A124	FJ569429	A23YM12RM	A139	FJ569527
A23YO05RM	A111	FJ569553	A23YH10RM	A125	FJ569434	A5YA07RM	LW80	FJ569836
A23YP10RM	A128	FJ569572	A23YH17RM	A126	FJ569440	A5YE18RM	LW81	FJ569937
A23YO02RM	EW94	FJ569551	A23YM19RM	A127	FJ569531	A5YG10RM	LW82	FJ569977
A6YD17RM	EW60	FJ570277	A24YM04RM	A129	FJ569776	A5YH18RM	LW83	FJ570008
A6YL11RM	EW61	FJ570460	A5YA09RM	LW76	FJ569838	A5YP06RM	LW85	FJ570176
A6YM09RM	EW62	FJ570481	A6YD18RM	EW70	FJ570278	A6YA13RM	EW77	FJ570205
A6YM10RM	EW63	FJ570482	A6YP20RM	EW71	FJ570560	A6YA19RM	EW78	FJ570211
A6YC15RM	EW69	FJ570251	A19YB03RM	P102	FJ568357	A6YE13RM	EW79	FJ570297
A21YC15RM	S101	FJ568826	A19YE18RM	P103	FJ568422	A6YI12RM	EW80	FJ570390
A19YD13RM	P83	FJ568399	A19YF10RM	P104	FJ568436	A19YI10RM	P124	FJ568489
A19YO05RM	P84	FJ568596	A19YG14RM	P105	FJ568457	A20YH22RM	P125	FJ568720
A19YO11RM	P85	FJ568602	A19YH16RM	P106	FJ568476	A21YH09RM	S158	FJ568919
A20YO11RM	P86	FJ568769	A19YI24RM	P107	FJ568499	A22YD05RM	S159	FJ569124
A21YI10RM	S108	FJ568937	A19YK04RM	P108	FJ568520	A22YO20RM	S160	FJ569274
A21YP21RM	S109	FJ569067	A21YA22RM	S131	FJ568792	A23YC09RM	A140	FJ569334
A23YA08RM	A112	FJ569290	A21YD06RM	S132	FJ568841	A24YB15RM	A141	FJ569604
A24YB19RM	A113	FJ569607	A21YJ20RM	S133	FJ568955	A24YC09RM	A142	FJ569618
A5YF15RM	LW70	FJ569958	A21YM23RM	S134	FJ569013	A24YC21RM	A143	FJ569625
A5YH17RM	LW71	FJ570007	A21YN10RM	S135	FJ569022	A24YD15RM	A144	FJ569641
A5YO16RM	LW72	FJ570163	A21YN24RM	S136	FJ569032	A24YE08RM	A145	FJ569654
A19YK11RM	P88	FJ568526	A24YD03RM	A130	FJ569630	A24YF09RM	A146	FJ569672
A23YE24RM	A114	FJ569385	A5YC21RM	LW77	FJ569893	A24YJ21RM	A147	FJ569742
A19YA15RM	P89	FJ568349	A5YM14RM	LW78	FJ570114	A24YM01RM	A148	FJ569773
A21YP10RM	S112	FJ569058	A5YM22RM	LW79	FJ570122	A24Yn04RM	A149	FJ569790
A23YE07RM	A116	FJ569370	A6YF20RM	EW72	FJ570328	A24Yn22RM	A150	FJ569802
A23YM05RM	A117	FJ569521	A6YH11RM	EW73	FJ570366	A19YP03RM	P128	FJ568613
A23Yn22RM	A118	FJ569548	A6YI22RM	EW74	FJ570399	A21YE05RM	S161	FJ568860
A24YP12RM	A119	FJ569825	A6YM19RM	EW75	FJ570491	A21YJ11RM	S162	FJ568950

A19YF09RM	P90	FJ568435	A6YM22RM	EW76	FJ570494	A23YF08RM	A152	FJ569392
A19YF21RM	P91	FJ568444	A19YC12RM	P126	FJ568380	A23YJ07RM	A153	FJ569468
A19YF22RM	P92	FJ568445	A19YC23RM	P111	FJ568389	A23YK08RM	A154	FJ569488
A19YG13RM	P93	FJ568456	A19YD08RM	P112	FJ568394	A23YO11RM	A156	FJ569558
A19YO02RM	P95	FJ568593	A19YF13RM	P113	FJ568439	A23YP02RM	A157	FJ569568
A21YB05RM	S113	FJ568798	A19YI05RM	P114	FJ568485	A24YA21RM	A158	FJ569595
A21YD15RM	S114	FJ568849	A19YI21RM	P115	FJ568497	A24YF06RM	A159	FJ569669
A21YI01RM	S115	FJ568929	A19YJ16RM	P116	FJ568510	A24YM10RM	A160	FJ569780
A21YK17RM	S116	FJ568971	A19YK16RM	P17	FJ568530	A22YD24RM	A161	FJ569137
A21YO06RM	S117	FJ569037	A19YK23RM	P118	FJ568536	A24YO09RM	A162	FJ569810
A21YO09RM	S118	FJ569039	A19YL17RM	P119	FJ568550	A5YB05RM	LW86	FJ569856
A22YA09RM	S119	FJ569073	A20YM11RM	P123	FJ568753	A5YL07RM	LW87	FJ570086
A23YD23RM	A120	FJ569363	A21YA18RM	S137	FJ568790	A5YL10RM	LW88	FJ570089
A23YG15RM	A121	FJ569416	A21YB11RM	S140	FJ568804	A5YM13RM	LW89	FJ570113
A5YE04RM	LW73	FJ569924	A21YB24RM	S141	FJ568813	A5YM18RM	LW90	FJ570118
A5YI17RM	LW74	FJ570027	A21YC03RM	S142	FJ568816	A6YA22RM	EW81	FJ570213
A5YK12RM	LW75	FJ570069	A21YC24RM	S143	FJ568835	A6YB11RM	EW82	FJ570226
A6YD13RM	EW65	FJ570273	A21YD05RM	S144	FJ568840	A6YC02RM	EW83	FJ570239
A6YG24RM	EW66	FJ570356	A21YE07RM	S145	FJ568861	A6YC05RM	EW84	FJ570242
A6YK08RM	EW67	FJ570433	A21YG11RM	S147	FJ568902	A6YE15RM	EW86AC	FJ570299
A19YA06RM	P96	FJ568343	A21YH08RM	S148	FJ568918	A6YE23RM	EW87	FJ570307
A19YC04RM	P97	FJ568374	A21YH14RM	S149	FJ568922	A6YG01RM	EW88	FJ570333
A19YC17RM	P98	FJ568383	A21YI20RM	S150	FJ568942	A6YH03RM	EW89	FJ570359
A19YI16RM	P99	FJ568493	A21YJ13RM	S151	FJ568951	A6YK05RM	EW90	FJ570430
A19YK14RM	P100	FJ568528	A21YJ15RM	S152	FJ568952	A6YL18RM	EW91	FJ570467
A21YA06RM	S120	FJ568783	A21YK23RM	S154	FJ568976	A6YO07RM	EW92	FJ570524
A21YA14RM	S121	FJ568787	A21YN13RM	S155	FJ569024	A6YO09RM	EW93	FJ570526
A21YB21RM	S122	FJ568811	A21YP05RM	S156	FJ569053	A21YP07RM	S105	FJ569055
A21YH15RM	S123	FJ568923	A21YP12RM	S157	FJ569059			
A21YD24RM	S124	FJ568855	A23YA05RM	A131	FJ569288			

C: α-proteobacteria

Sample	Code	Accession number	Sample	Code	Accession number	Sample	Code	Accession number
A19YA02RM	P1	FJ568340	A21YO20RM	S34	FJ569046	A24YL18RM	A50	FJ569770
A19YA10RM	P2	FJ568345	A21YP06RM	S35	FJ569054	A24YN15RM	A51	FJ569796
A19YC19RM	P3	FJ568385	A21YP19RM	S36	FJ569065	A24YO01RM	A52	FJ569804
A19YD21RM	P4	FJ568406	A21YP20RM	S37	FJ569066	A24YO16RM	A53	FJ569815
A19YE11RM	P5	FJ568418	A21YP23RM	S38	FJ569068	A24YP23RM	A54	FJ569832
A19YE20RM	P6	FJ568424	A22YA20RM	S39	FJ569082	A5YB22RM	LW2	FJ569871
A19YF16RM	P7	FJ568441	A22YB05RM	S40	FJ569089	A5YD13RM	LW5	FJ569909
A19YG15RM	P8	FJ568458	A22YD01RM	S43	FJ569121	A5YD18RM	LW6	FJ569914
A19YH03RM	P9	FJ568468	A22YD14RM	S44	FJ569130	A5YD19RM	LW7	FJ569915
A19YI06RM	P10	FJ568486	A22YD22RM	S45	FJ569136	A5YE13RM	LW8	FJ569933
A19YJ17RM	P11	FJ568511	A22YJ04RM	S47	FJ569201	A5YF11RM	LW9	FJ569954
A19YK02RM	P12	FJ568518	A22YK24RM	S48	FJ569224	A5YG07RM	LW10	FJ569974
A19YL03RM	P14	FJ568539	A22YM15RM	S49	FJ569243	A5YG13RM	LW11	FJ569979
A19YL09RM	P15	FJ568559	A23YA04RM	A2	FJ569287	A5YG18RM	LW12	FJ569984

A19YM02RM	P16	FJ568560	A23YA21RM	A3	FJ569304	A5YH07RM	LW13	FJ569997
A19YM03RM	P17AL	FJ568566	A23YA23RM	A4	FJ569303	A5YH11RM	LW15	FJ570001
A19YM10RM	P18	FJ568581	A23YA24RM	A5	FJ569304	A5YH13RM	LW16	FJ570003
A19YN07RM	P19	FJ568582	A23YH03RM	A6	FJ569428	A5YH14RM	LW17	FJ570004
A19YN08RM	P20	FJ568603	A23YC11RM	A7	FJ569336	A5YH24RM	LW18	FJ570013
A20YP07RM	P21	FJ568775	A23YD16RM	A9	FJ569357	A5YJ05RM	LW19	FJ570038
A19YP21RM	P22	FJ568627	A23YD20RM	A10	FJ569361	A5YJ06RM	LW20	FJ570039
A20YA09RM	P23	FJ568633	A23YE14RM	A11	FJ569376	A5YK01RM	LW21	FJ570058
A20YJ01RM	P26	FJ568725	A23YE18RM	A12	FJ569380	A5YM04RM	LW24	FJ570104
A20YK05RM	P27	FJ568734	A23YG01RM	A14	FJ569405	A5YM07RM	LW25	FJ570107
A20YK07RM	P28	FJ568735	A23YG05RM	A15	FJ569409	A5YM19RM	LW26	FJ570119
A21YB03RM	S1	FJ568797	A23YG16RM	A16	FJ569417	A5YM23RM	LW27	FJ570123
A21YB06RM	S2	FJ568799	A23YG20RM	A17	FJ569421	A5YM24RM	LW28	FJ570124
A21YC10RM	S4	FJ568821	A23YH03RM	A18	FJ569428	A5YN03RM	LW29	FJ570127
A21YC11RM	S5	FJ568822	A23YH07RM	A19	FJ569431	A5YN15RM	LW30	FJ570138
A21YC16RM	S6	FJ568827	A23YH18RM	A20	FJ569441	A5YP11RM	LW31	FJ570181
A21YD03RM	S7	FJ568838	A23YI01RM	A21	FJ569447	A6YA01RM	EW2	FJ570193
A21YD16RM	S8	FJ568850	A23YI08RM	A22	FJ569452	A6YB01RM	EW3	FJ570216
A21YD18RM	S9	FJ568851	A23YI12RM	A23	FJ569455	A6YC18RM	EW6	FJ570254
A21YD20RM	S10	FJ568852	A23YJ01RM	A24	FJ569464	A6YD08RM	EW8	FJ570268
A21YD22RM	S11	FJ568854	A23YJ05RM	A25	FJ569466	A6YD09RM	EW9	FJ570269
A21YE02RM	S12	FJ568857	A23YJ12RM	A26	FJ569472	A6YE01RM	EW10	FJ570285
A21YE09RM	S13AL	FJ568862	A23YJ13RM	A27	FJ569473	A6YE05RM	EW11	FJ570289
A21YF04RM	S14	FJ568879	A23YK12RM	A28	FJ569492	A6YE18RM	EW12	FJ570302
A21YF06RM	S15	FJ568881	A23YK19RM	A29	FJ569498	A6YE20RM	EW13	FJ570304
A21YF19RM	S16	FJ568890	A23YK23RM	A30	FJ569501	A6YF04RM	EW14	FJ570312
A21YF24RM	S17	FJ568895	A23YL10RM	A31	FJ569509	A6YF24RM	EW15	FJ570332
A21YG09RM	S18	FJ568901	A23YM08RM	A33	FJ569524	A6YG21RM	EW16	FJ570353
A21YG16RM	S19	FJ568906	A23YO23RM	A34	FJ569565	A6YG22RM	EW17	FJ570354
A21YH01RM	S20	FJ568914	A23YO01RM	A35	FJ569550	A6YH20RM	EW18	FJ570375
A21YH03RM	S21	FJ568916	A24YB16RM	A36	FJ569605	A6YI24RM	EW20	FJ570401
A21YI09RM	S22	FJ568936	A24YB22RM	A37	FJ569610	A6YJ04RM	EW21	FJ570405
A21YI12RM	S23	FJ568938	A24YC14RM	A38	FJ569620	A6YJ19RM	EW22	FJ570420
A21YI24RM	S24	FJ568945	A24YG03RM	A41	FJ569685	A6YJ24RM	EW23	FJ570425
A21YL16RM	S26	FJ568987	A24YG15RM	A42	FJ569697	A6YK17RM	EW25	FJ570442
A21YL24RM	S27	FJ568993	A24YI01RM	A43	FJ569717	A6YL08RM	EW26	FJ570457
A21YM03RM	S28	FJ568996	A24YI12RM	A44	FJ569723	A6YL21RM	EW27	FJ570470
A21YM19RM	S30	FJ569009	A24YJ16RM	A45	FJ569739	A6YM07RM	EW28	FJ570479
A21YN03RM	S31	FJ569016	A24YJ23RM	A46	FJ569744	A6YN09RM	EW30	FJ570504
A21YN05RM	S32	FJ569018	A24YK11RM	A47	FJ569752	A6YN10RM	EW31	FJ570505
A21YO19RM	S33	FJ569045	A24YL01RM	A48	FJ569761	A6YO03RM	EW32	FJ570520

D: β, γ and δ-proteobacteria

Sample	Code	Accession number	Sample	Code	Accession number	Sample	Code	Accession number
A19YH12RM	P30	FJ568474	A6YH19RM	A85	FJ570374	A6YE09RM	EW46	FJ570293
A19YH22RM	P31	FJ568481	A6YL19RM	EW39	FJ570468	A6YF18RM	EW47	FJ570326
A19YO17RM	P32	FJ568607	A6YM06RM	EW40	FJ570478	A6YI09RM	EW48	FJ570387

A20YA21RM	P33	FJ568639	A6YM20RM	EW41	FJ570492	A6YL04RM	EW49	FJ570453
A20YB03RM	P34	FJ568643	A6YP02RM	EW42	FJ570543	A6YL06RM	EW50	FJ570455
A20YC23RM	P35	FJ568668	A6YP12RM	EW43	FJ570553	A6YO11RM	EW51	FJ570528
A20YE08RM	P36	FJ568688	A19YA14RM	P41	FJ568347	A19YC02RM	P60	FJ568373
A21YB22RM	S57	FJ568812	A19YA13RM	P42	FJ568348	A19YC20RM	P61	FJ568386
A21YC07RM	S58	FJ568819	A19YC16RM	P43	FJ568382	A19YD16RM	P62	FJ568402
A21YE13RM	S59	FJ568866	A19YC21RM	P44	FJ568387	A19YD19RM	P63	FJ568404
A21YF01RM	S60	FJ568876	A19YC24RM	P45	FJ568390	A19YD24RM	P64	FJ568409
A21YF12RM	S61	FJ568885	A19YD09RM	P46	FJ568395	A19YG19RM	P65	FJ568461
A21YG01RM	S62	FJ568896	A19YE03RM	P47	FJ568411	A19YG20RM	P66	FJ568462
A21YG12RM	S63	FJ568903	A19YE09RM	P48	FJ568416	A19YH02RM	P67	FJ568484
A21YG19RM	S64	FJ568908	A19YF05RM	P49	FJ568431	A19YI04RM	P68	FJ568496
A21YJ21RM	S65	FJ568956	A19YG06RM	P50	FJ568451	A19YI20RM	P69	FJ568496
A21YL02RM	S66	FJ568978	A19YG08RM	P51	FJ568452	A19YJ19RM	P70	FJ568512
A21YL15RM	S67	FJ568986	A19YK24RM	P52	FJ568537	A19YL16RM	P71	FJ568549
A21YN04RM	S68	FJ569017	A19YN17RM	P53	FJ568589	A19YM01RM	P72	FJ568558
A21YN06RM	S69	FJ569019	A19YN24RM	P54	FJ568592	A19YM09RM	P73	FJ568565
A21YN09RM	S70	FJ569021	A20YM13RM	P55	FJ568754	A19YM22RM	P74	FJ568576
A21YP24RM	S71	FJ569069	A20YN09RM	P56	FJ568760	A19YN15RM	P75	FJ568587
A22YB14RM	S72	FJ569096	A21YB01RM	S77	FJ568795	A19YP01RM	P76	FJ568612
A22YE01RM	S73	FJ569138	A21YD02RM	S78	FJ568837	A19YP14RM	P77	FJ568621
A22YF17RM	S74	FJ569161	A21YG23RM	S79	FJ568912	A20YM10RM	P78	FJ568752
A23YB09RM	A60	FJ569312	A21YJ10RM	S80	FJ568949	A21YC18RM	S93	FJ568829
A23YB13RM	A61	FJ569315	A21YK12RM	S81	FJ568967	A21YD14RM	S94	FJ568848
A23YB16RM	A62	FJ569318	A21YN02RM	S82	FJ569015	A21YF14RM	S95	FJ568886
A23YB22RM	A63	FJ569324	A21YN15RM	S83	FJ569026	A21YH02RM	S96	FJ568915
A23YC02RM	A64	FJ569327	A21YN23RM	S84	FJ569031	A21YK03RM	S97	FJ568961
A23YC22RM	A65	FJ569343	A21YP08RM	S85	FJ569056	A21YL06RM	S98	FJ568982
A23YE02RM	A66	FJ569366	A22YF09RM	S86	FJ569157	A21YL11RM	S99	FJ568984
A23YE15RM	A67	FJ569377	A22YG05RM	S87	FJ569167	A21YL18RM	S100	FJ568988
A23YE22RM	P38	FJ569383	A22YK13RM	S88	FJ569219	A21YM02RM	S101	FJ568995
A23YF05RM	A68	FJ569389	A22YM22RM	S89	FJ569245	A21YO05RM	S102	FJ569036
A23YH08RM	A69	FJ569432	A22YM23RM	S90	FJ569246	A22YE06RM	S103	FJ569142
A23YH12RM	A70	FJ569436	A22YN02RM	S91	FJ569249	A23YA03RM	A100DE	FJ569286
A23YL15RM	A71	FJ569512	A22YN07RM	S92	FJ569253	A23YB24RM	A101	FJ569325
A19YH05RM	P39	FJ568469	A23YA20RM	A88	FJ569300	A23YD06RM	A102DE	FJ569351
A23YN21RM	A72	FJ569547	A23YC18RM	A89	FJ569341	A23YF19RM	A103DE	FJ569400
A23YO20RM	A73	FJ569562	A23YD24RM	A90	FJ569364	A23YH02RM	A104	FJ569427
A23YP01RM	A74	FJ569567	A23YE17RM	A91	FJ569379	A23YH09RM	A105	FJ569433
A23YP03RM	A75	FJ569569	A23YI09RM	A92	FJ569453	A23YK06RM	A106	FJ569486
A23YP23RM	A76	FJ569581	A23YK16RM	A93	FJ569495	A23YK17RM	A107	FJ569496
A24YC07RM	A77	FJ569616	A23YO19RM	A94	FJ569561	A5YC14RM	LW55	FJ569886
A24YE17RM	A78	FJ569659	A23YP21RM	A95	FJ569579	A5YD04RM	LW56	FJ569900
A24YI08RM	A79	FJ569719	A24YA13RM	A96	FJ569590	A5YD20RM	LW57	FJ569916
A24YJ05RM	A80	FJ569731	A24YE03RM	A97	FJ569649	A5YD22RM	LW58	FJ569918
A24YJ09RM	A81	FJ569735	A24YE07RM	A98	FJ569653	A5YE08RM	LW59	FJ569928
A24YK22RM	A82	FJ569759	A24YG14RM	A99	FJ569415	A5YE19RM	LW60	FJ569938
A24YM03RM	A83	FJ569775	A24YH11RM	A100	FJ569710	A5YI23RM	LW61	FJ570032
A24YM22RM	A84	FJ569786	A24YH22RM	A101DE	FJ569716	A5YJ10RM	LW62	FJ570043
A24YP03RM	A85	FJ569819	A24YI10RM	A102	FJ569721	A5YK06RM	LW63	FJ570063

A24YP22RM	A86	FJ569831	A24YO23RM	A103	FJ569818	A5YM15RM	LW64	FJ570115
A5YA21RM	LW35	FJ569849	A5YC19RM	LW43	FJ569891	A5YO09RM	LW65	FJ570156
A5YB10RM	P40	FJ569861	A5YF23RM	LW44	FJ569966	A5YP07RM	LW66	FJ570177
A5YE22RM	LW36	FJ569941	A5YH06RM	LW45	FJ569996	A6YB04RM	EW52	FJ570219
A5YF08RM	LW37	FJ569951	A5YH20RM	LW47	FJ570010	A6YD10RM	EW53	FJ570270
A5YH16RM	LW38	FJ570006	A5YJ02RM	LW48	FJ570035	A6YG12RM	EW54	FJ570344
A5YI15RM	LW39	FJ570025	A5YJ11RM	LW49	FJ570044	A6YG18RM	EW55	FJ570350
A5YJ18RM	LW40	FJ570051	A5YN16RM	LW50	FJ570139	A6YG19RM	EW56	FJ570351
A5YN17RM	LW41	FJ570140	A5YO12RM	LW51	FJ570159	A6YJ13RM	EW57	FJ570414
A6YA03RM	EW34	FJ570195	A5YO15RM	LW52	FJ570162	A6YK04RM	EW58	FJ570429
A6YB16RM	EW35	FJ570230	A5YP02RM	LW53	FJ570172	A6YN22RM	EW59	FJ570517
A6YB23RM	EW36	FJ570236	A5YP03RM	LW54	FJ570173			
A6YF12RM	EW37	FJ570320	A6YC12RM	EW45	FJ570249			

Annexe C

Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach

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Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach

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Summary

In alpine ecosystems, tannin-rich-litter decomposition occurs mainly under snow. With global change, variations in snowfall might affect soil temperature and microbial diversity with biogeochemical consequences on ecosystem processes. However, the relationships linking soil temperature and tannin degradation with soil microorganisms and nutrients fluxes remain poorly understood. Here, we combined biogeochemical and molecular profiling approaches to monitor tannin degradation, nutrient cycling and microbial communities (Bacteria, Crenarcheotes, Fungi) in undisturbed wintertime soil cores exposed to low temperature (0°C/–6°C), amended or not with tannins, extracted from *Dryas octopetala*. No toxic effect of tannins on microbial populations was found, indicating that they withstand phenolics from alpine vegetation litter. Additionally at –6°C, higher carbon mineralization, higher protocatechuic acid concentration (intermediary metabolite of tannin catabolism), and changes in fungal phylogenetic composition showed that freezing temperatures may select fungi able to degrade *D. octopetala*'s tannins. In contrast, negative net nitrogen mineralization rates were observed at –6°C possibly due to a more efficient N immobilization by tannins than N production by microbial activities, and suggesting a decoupling between C and N mineralization. Our results confirmed tannins and soil temperatures as relevant controls of microbial catabolism which are crucial for alpine ecosystems functioning and carbon storage.

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Introduction

In arctic and alpine ecosystems, seasonally snow-covered soils sequester a very large pool of organic carbon, which appears particularly vulnerable in the context of global warming (Hobbie *et al*., 2000). A positive feed-back between increased soil respiration and rising atmospheric CO_2 has been put forward several times in global carbon balance models (Knorr *et al*., 2005). However, whether snow-covered ecosystems are carbon sources or sinks is still highly debated (Mack *et al*., 2004; Knorr *et al*., 2005). This is partly explained by the incomplete understanding we have of the processes involved in the wintertime heterotrophic respiration in relation to snow cover duration in cold ecosystems (McGuire *et al*., 2000; Monson *et al*., 2006). The variations of soil microbial activity in relation with the dynamic of the snow cover and litter inputs are poorly documented, though some seasonal changes in the structure and function of microbial communities in alpine soils have been described (Lipson *et al*., 2002; Schadt *et al*., 2003; Lipson and Schmidt, 2004). It has been shown that the cooler temperature at the end of the growing season triggers a marked shift to a psychrophilic microflora dominated by fungi (Lipson *et al*., 2002). Additionally, the microbial biomass increased sharply during wintertime. These microbial communities are able to decompose efficiently recalcitrant carbon sources, such as polyphenols, which are likely to be abundant in alpine plants tissues (Steltzer and Bowman, 2005). It is widely recognized that phenolics play a major role in nutrient cycling and litter decomposition through their multilevel interactions with mineralization processes (Cornelissen *et al*., 1999; Hattenschwiler and Vitousek, 2000). Aside from their toxicity towards some microorganisms, polyphenols, especially the tannin fraction, are expected to affect the availability of nitrogen to plants during their growing season, mainly through complexation

of the organic nitrogen in soils (Kraus *et al*., 2003; Kaal *et al*., 2005; Nierop and Verstraten, 2006).

Fungus-dominated microbial communities are particularly abundant in the most constraining habitats of the alpine landscape such as exposed dry meadows (Nemergut *et al*., 2005). These ecosystems are dominated by slow-growing plant species – mainly *Kobresia myosuroides*, *Dryas octopetala* – and are characterized by a low net primary productivity, a high soil organic matter (SOM) content, and a limited supply of soil nutrients (Choler, 2005). Furthermore, *D. octopetala* produces high amounts of polyphenols, with proanthocyans as the major tannin compounds. In dry meadows, the low and irregular snow pack leads to frequent periods where soils are frozen (< –5°C) between plant senescence and renewal. However, the impact of repeated low temperature events on both recalcitrant litter decomposition and soil functioning remains unknown. Additionally, these abiotic constraints are likely to be modified by climatic change. Recent climate scenarios for the Alps show changes in the seasonality and quantity of snow at high altitude, i.e. above 2000 m (Beniston, 2003; Keller *et al*., 2005). The predicted decrease in precipitation between autumn and early spring will most likely reduce the winter snow-covered period of alpine dry meadows, consequently, increasing the length of the soil freezing period. It is not known to what extent these changes will affect the wintertime decomposition of organic matter, recalcitrant compounds in particular.

In this study, we focused on the combined effect of low temperature (< 0°C) and the input of recalcitrant compounds on alpine soil functioning. We expected a shift in microbial communities as a consequence of changes in these two ecological factors during the late-fall critical period. We set up an incubation experiment with soil cores under laboratory conditions to disentangle the effects of temperature and tannin addition on the diversity of microbial communities and the carbon and nitrogen cycles. More specifically, we addressed the following questions: how does tannin input affect (i) carbon and nitrogen mineralization and (ii) overall soil bacterial and fungal phylogenetic structures? (iii) how are these functional and phylogenetic responses are modulated by a prior treatment at freezing temperature (–6°C)?

We simulated a late-fall litter flux by adding tannins extracted from *D. octopetala* leaves to soil cores collected in dry meadows during the fall, and we mimicked the snow-pack reduction by a freezing treatment. We monitored the C and N soil dynamics (including tannin evolution) and assessed the microbial soil diversity through rRNA genes (16S rRNA gene for prokaryotes, ITS for fungi) using molecular profiling [single-strand conformation polymorphism (SSCP)] in addition to classical microbial techniques.

Results

Impact of tannin on structure and metabolism of microbial populations were addressed in an incubation experiment with soil cores under laboratory conditions. Four treatments were applied ($n = 3$). In W/S and T/S treatments, soil cores were amended, respectively, with water and tannins extracted from *D. octopetala* leaves, and they were all maintained at 0°C during 45 days. In W/F and T/F, soil cores were also amended with water and tannin solution respectively, but then were stored at –6°C during 15 days (day 15) and kept at 0°C for four more weeks.

Phenolic metabolization

At day 15, more than 10% of the added tannins were recovered from the soil samples, 12% for S/T (Stable/Tannin treatment) and 17% for F/T (Freezing/Tannin treatment) (non-significant difference, $U = 2.5$, $P = 0.376$). After 45 days, both temperature treatments had a recovery fraction of around 5%. In treatment W, no tannins were detected in the soils at days 15 and 45, while they were present in low but detectable amounts at day 0. When comparing the phenolic acids, significantly higher levels of protocatechuic acid (last aromatic degradation metabolite before ring fission) were observed in the treatment T, than in the treatment W, irrespective of temperature and sampling times (Fig. 1). The accumulation of this degradation product was higher in the F/T treatment than in the S/T treatment for both dates, indicating a better metabolizing of the tannins in soils submitted to the freezing treatment. Similar patterns were observed for other phenolic acids: vanillic and p-hydroxybenzoic acids (data not shown).

Changes in microbial biomass and diversity

Microbial and fungal biomasses and bacterial counts were not significantly affected by temperature or tannin amendment (Table 1). However, these treatments affected microbial diversity differently. The F/T and S/W treatments had the strongest impact on the bacterial communities (Fig. 2A). Moreover marked differences between day 15 and day 45 are supported for all the treatments except for F/W.

For crenarcheotes, we observed the formation of new SSCP peaks for all the treatments (data not shown). The F/T treatment had a contrasted effect on the structure (peak distribution) of the crenarcheote communities compared with other conditions (data not shown) as there were less peaks, which suggests the dominance of few phylotypes.

Diversity of fungal communities was significantly affected for all conditions and especially for S/T and F/W. The SSCP profile of day 45 (F/T) was an outlier because

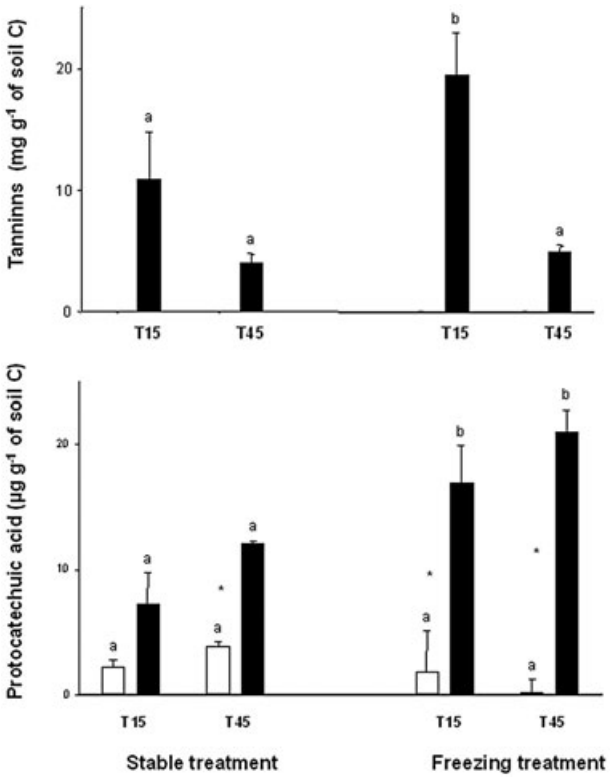

Fig. 1. A. Average tannins concentrations in soil amended with sterilized water (white bars) or a tannin solution (black bars). The concentration of tannins is negligible in the case of sterilized water amendment.
B. Protocatechuic acid concentration in soils amended with sterilized water (white bars) or a tannin solution (black bars). Differences between water and tannin treatments (*$P < 0.05$) and between temperature treatments (a, b; $P < 0.05$) were tested with Mann–Whitney tests. $n = 3 \pm$ standard error of the mean.

the data file containing the migration value for statistical analysis was corrupted. The longest distance corresponded to the F/W cores, which showed fewer peaks than the other treatments (Fig. 3D). The day 0 and S/W profiles (Fig. 3A and B) had more than 10 peaks. The S/T profiles (Fig. 3C) presented a low signal, but as many peaks as S/W and day 0. A broad analysis of the raw data for S/T revealed that the baseline increased, which may indicate the co-migration of numerous fungal phylotypes (Loisel *et al.*, 2006). For all profiles, the predominant phylotypes became relatively more abundant between days 15 and 45. Moreover, the F/T profiles presented more peaks than the S/T ones (Fig. 3E). These results suggest that tannins prevented the loss of fungal phylotypes due to freezing.

Impact on carbon mineralization

Between days 0 and 15, the total CO_2 efflux measured with the F treatment was significantly lower (approximately two- to fourfold) than with the S treatment (Table 2). Although marginally significant, the tannin treatment (F/T) led to an increase (approximately twofold) in CO_2 efflux between days 0 and 15 compared with the F/W treatment. However, the total CO_2 efflux at 0°C was not affected by the presence of tannins.

On day 15, when the temperature shifted from –6°C to 0°C, the CO_2 efflux doubled from soils of F/T treatment and increased fourfold in the F/W treatment. Between days 15 and 45, no additional differences were detected between the treatments with and without tannins (Table 2). These results indicated that tannins enhanced the CO_2 efflux only with the freezing treatment between days 0 and 15.

Impact on nitrogen cycling

The nitrogen dissolved in the soil extracts was mainly in organic forms [~529.6–1416.7 µg N g^{-1} dry weight (dw), 90.4–99.6% of total dissolved nitrogen (TDN)], while ammonia (~3.2–67.1 µg N g^{-1} dw, 0.3–8.7% of TDN) and nitrate/nitrite (0.1–10.2 µg N g^{-1} dw, 0.0–1.3% of TDN) made up smaller proportions of the TDN. Total dissolved nitrogen and dissolved organic nitrogen (DON) soil contents were changed neither by the temperature (F versus S treatments, data not shown) nor by the addition of tannins (T versus W treatments). Within S treatment, net N mineralization rates between days 15 and 45 were not influenced by the presence of tannins, whereas they were significantly reduced in soils previously stored at –6°C (F treatment, Fig. 4A). This effect was even stronger in soils in the F/T treatment, for which we measured net N immobilization values suggesting that the production of inorganic N was not sufficient to compensate for its disappearance.

Net mineralization potentials (NMP) measured on soil subsamples at days 0, 15 and 45 were 10–400 times

Table 1. Microbial and fungal biomasses and bacterial count estimated from soil cores on days 15 and 45 ($n = 3$).

Treatments	Microbial biomass (mg C g^{-1} C)	Fungal biomass (µg ergosterol g^{-1} C)	Bacterial count (10^9 cells g^{-1} C)
T15			
S/W	44.9 (11.9)^{aA}	60.9 (12.6)^{aA}	1.13 (0.13)^{aA}
F/W	45.8 (15.5)^{aA}	182.1 (38.1)^{aA}	1.46 (0.28)^{aA}
S/T	35.3 (8.6)^{aA}	53.8 (5.2)^{aA}	1.01 (0.06)^{aA}
F/T	38.0 (17.0)^{aA}	94.1 (37.3)^{aA}	1.12 (0.20)^{aA}
T45			
S/W	16.6 (2.2)^{aA}	114.3 (18.1)^{aA}	1.40 (0.34)^{aA}
F/W	48.1 (5.3)^{aA}	232.0 (42.7)^{aA}	1.59 (0.45)^{aA}
S/T	18.7 (6.3)^{aA}	103.8 (20.3)^{aA}	1.19 (0.50)^{aA}
F/T	19.3 (7.1)^{aA}	117.2 (35.5)^{aA}	0.89 (0.06)^{aA}

Differences between day 15 and day 45 were tested by Wilcoxon signed rank test (upper case, $P < 0.05$) and differences between treatment (temperature or tannin amendment) by Mann–Whitney rank sum test (lower case, $P < 0.05$). $n = 3 \pm$ standard error of the mean.

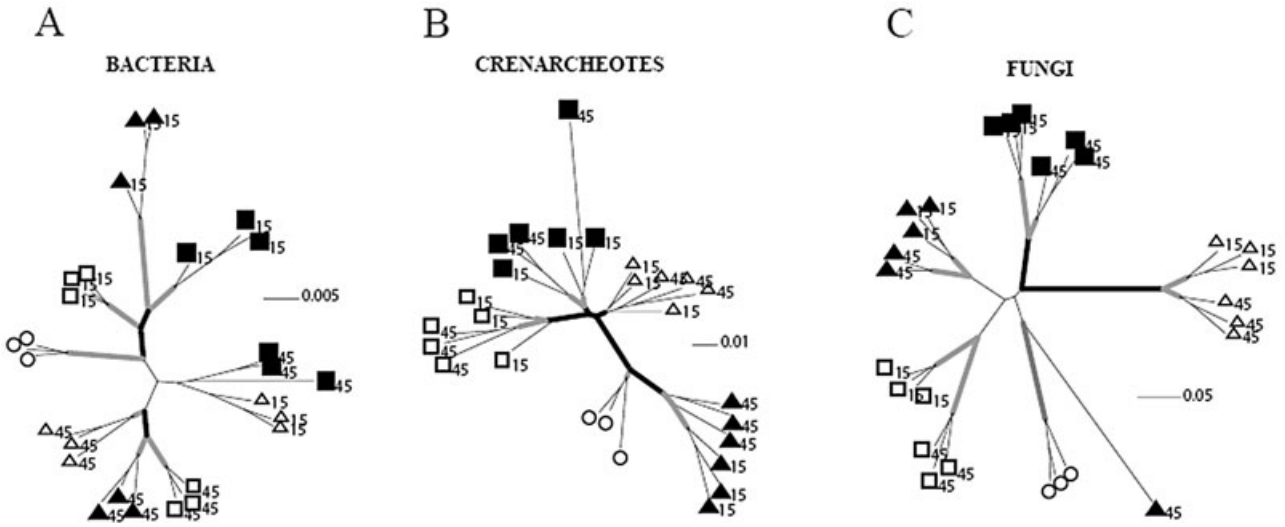

Fig. 2. Neighbour-Joining trees of microbial communities under different treatments: ○= day 0; □= S/W; ■= S/T, △= F/W; ▲= F/T. Lower cases indicate the day of treatments. Supported branches (bootstrap value >50%) are in bold. Grey corresponds to the SSCP replicates after pooling the three soil cores from the same treatment. Black corresponds to supported clustering of treatments. Edwards's distances are shown by a scale bar.

higher than the net N mineralization measured between days 15 and 45 (Fig. 4B and C). Yet, NMP increased during the incubation in the case of the F/W treatment but not in the F/T treatment. On days 15 and 45, NMP from soils amended with tannins were significantly lower than for the unamended ones (Fig. 4B). In treatment S, soils incubated with tannins (S/T) had also lower NMP than on soils amended with water (S/W) but only on day 15 (Fig. 4C). Net mineralization potentials were therefore strongly reduced in F/T treatment compared with the others.

Discussion

In our study, the role of tannins was evaluated through the combination of biogeochemical analyses with molecular profiling approach. The few other studies which examined the impact of polyphenols through purified tannin addition focused on forested ecosystems, characterized by faster nutrient cycling and higher productivity (Bradley *et al*., 2000; Fierer *et al*., 2001). Furthermore, unlike those studies, we used undisturbed soil cores instead of composite and homogenized samples to maintain the vertical stratification and its associated physical and microbiological properties.

Impact on carbon and nitrogen cycles

The organic and inorganic nitrogen soil concentrations measured in the alpine soils, as well as the dominance of organic N forms, were in accordance with the literature (Tosca and Labroue, 1986; Lipson *et al*., 1999; Zeller *et al*., 2000). Similarly, CO_2 efflux measurements were within the range of previous observations (Leifeld and Fuhrer, 2005; Schimel and Mikan, 2005). After 1 month, only a minor fraction of the added tannins was recovered from the amended soils. The disappearance of tannins could be explained either by degradation or by insolubilization, due to complexation with proteins or adsorption on organo-mineral soil fractions (Kaal *et al*., 2005; Nierop and Verstraten, 2006).

In stable treatment, tannin addition had limited effects on C and N mineralization as well as on microbial biomass indicating (i) that tannins were not used as a significant extra C source and (ii) that they did not inhibit microbial communities. The slight increase in protocatechuic acid

Table 2. Soil water content, daily mean CO_2 efflux over the days 0–15 and days 15–45.

Treatments	Soil water content (%)	CO_2 efflux ($\mu g\ C\ g^{-1}\ C\ day^{-1}$)
T0-T15		
S/W	33.4 (1.0)^{aA}	318.8 (70.4)^{aA}
F/W	34.8 (3.8)^{aA}	76.7 (7.8)^{bA}
S/T	37.6 (4.1)^{aA}	267.9 (71.9)^{abA}
F/T	30.9 (4.5)^{aA}	128.6 (19.2)^{abA}
T15-T45		
S/W	33.2 (1.2)^{aA}	281.3 (131.0)^{aA}
F/W	34.9 (3.1)^{aA}	269.4 (51.5)^{aB}
S/T	39.1 (4.2)^{aA}	210.7 (23.7)^{aA}
F/T	34.0 (4.7)^{aA}	273.8 (94.5)^{aB}

The differences between days 15 and 45 within each treatment (upper case, $P < 0.05$) were tested by Wilcoxon signed rank test and the differences between treatment (temperature or tannin addition) within each period (lower case, $P < 0.05$) were tested by Mann–Whitney rank sum test. $n = 3 \pm$ standard error of the mean.

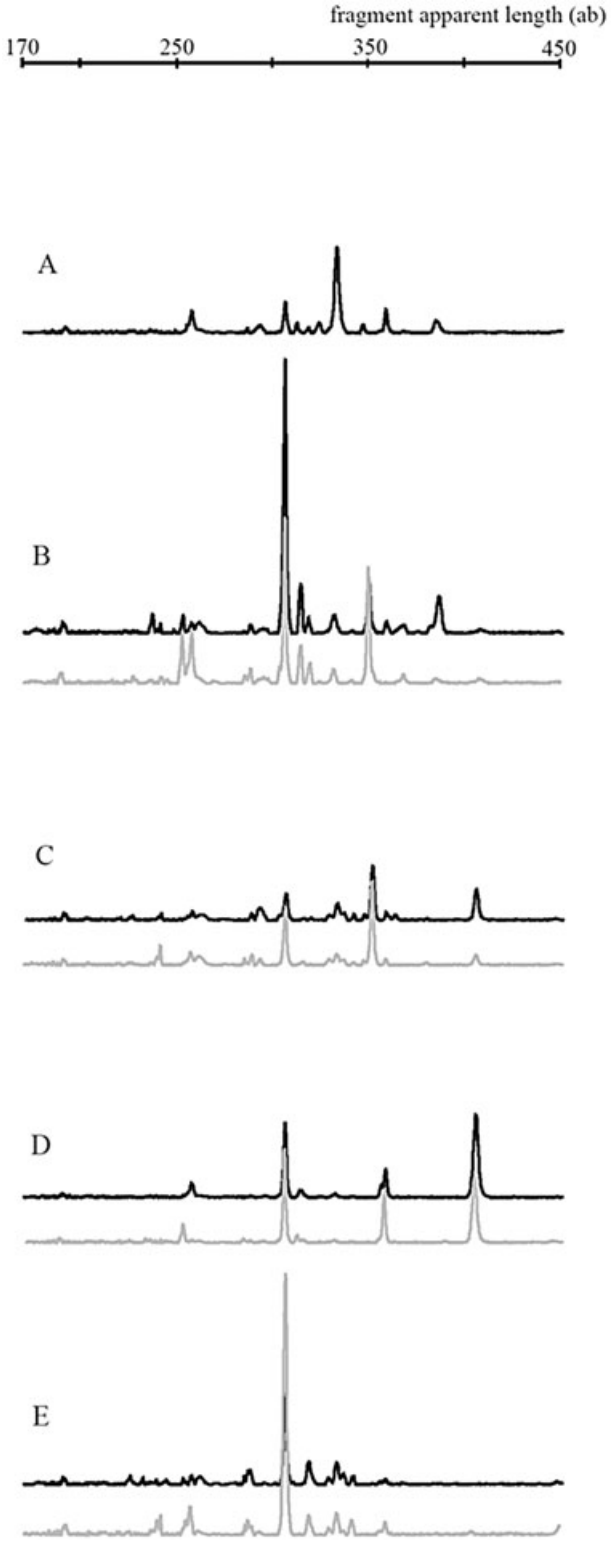

Fig. 3. Capillary electrophoresis-SSCP profiles of fungal communities for each treatment. A = day 0; B = S/W; C = S/T; D = F/W; E = F/T. Black lines: day 15; grey lines: day 45. All profiles are displayed for an arbitrary fluorescence intensity interval of 4000.

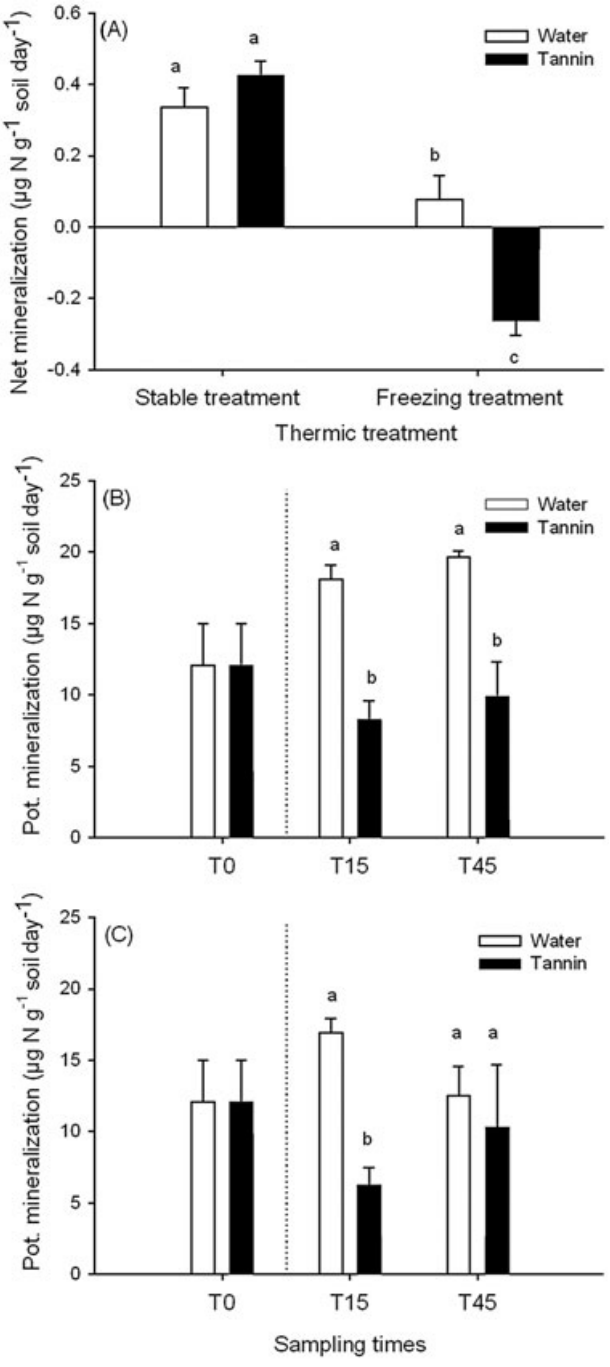

Fig. 4. A. Net N mineralization rates between days 15 and 45 in soils for F and S treatments amended with sterilized water (white bars) or a tannin solution (black bars).
B and C. Net N mineralization potentials in soils after 0, 15 and 45 days of incubation in the F treatment (B) and the S treatment (C), amended with sterilized water (white bars) or a tannin solution (black bars). Mann–Whitney rank sum tests were performed to test for differences between treatment (temperature or tannin addition) within each period (lower case, $P < 0.05$). $n = 3 \pm$ standard error of the mean.

indicated only weak tannin degradation. This suggests that tannins were preferentially adsorbed on organo-mineral soils or complexed with decomposing organic compounds (Fierer *et al*., 2001). But this hypothesis, largely mentioned in the literature, is hypothetical as efficient methods to quantify such insoluble compounds are missing (Lorenz *et al*., 2000; Kanerva *et al*., 2006). Also, we did not assess the impact of a prolonged exposure to tannins as it occurs in natural habitats, but rather try to mimic a pulse of tannins corresponding to the litter fall period. Thus, it should not be considered as a long-term effect.

The incubation of alpine soils at –6°C led to a decrease in C mineralization between day 0 and day 15. Lower temperatures strongly affect soil processes by lowering microbial enzymatic activities (Schimel *et al*., 2004) but do not affect microbial biomass as already shown by others authors (Lipson *et al*., 2000; Groffman *et al*., 2001; Grogan *et al*., 2004). Surprisingly, higher CO_2 effluxes were detected in F/T than in F/W treatments and higher concentrations of protocatechuic acid in F/T than in S/T treatments in the first step of the experiment. These results indicate a higher tannin degradation at –6°C. Added to limited microbial growth, this suggests that some microorganisms may be able to use this C source, unlike the populations present at 0°C (S/T). However, the lack of data on N mineralization between day 0 and day 15 prevents us from drawing further conclusions.

From day 15 to day 45, the only significantly affected process was net N mineralization, which decreased in F/W-treated alpine soils. This confirms that a prolonged pre-period of frost slowed down N cycling in alpine soils (Schimel *et al*., 2004). The reduction of metabolic activities in the soil microbial community may be responsible for this effect. The addition of tannins in our alpine soils amplified the freezing effect on net N mineralization described previously and even led to apparent N immobilization (F/T, Fig. 4A). Possibly, the complexing capacity of tannins may have been detected only when microorganisms were less active due to freezing, suggesting that the kinetics of N mineralization by living microorganisms was faster than that of abiotic N immobilization by tannins. Both processes occurred in the stable treatment, but N microbial mineralization was dominant and microorganisms transformed N_{org} into NH_4^+ and NO_3^- in much larger quantities than could be complexed by tannins. As a result, we measured no tannin effect on net N mineralization in soils for the S treatment (measured as the amount of NH_4^+ and NO_3^- produced).

In F treatment, the reduction of microbial activities reduced the production of NH_4^+ and NO_3^-, which did not compensate for the complexation by tannins (Fierer *et al*., 2001; Castells *et al*., 2003). In our experiment, this was perceived as a negative net N mineralization or an apparent NH_4^+ and NO_3^- immobilization. This hypothesis is further supported by NMP results (at 30°C) which were significantly lower in soils amended with tannins, most probably due to complexation of organic compounds (Fierer *et al*., 2001).

The high concentration of protocatechuic acid and C mineralization during the first step of freezing treatment suggests that the added tannins were metabolized despite very low temperatures. C mineralization was strongly affected by the temperature shift and no long-term effect of tannin addition was detected, possibly due to the shortage of easily decomposable tannin (Kraus *et al*., 2004). The absence of relationship between N immobilization and C mineralization between days 15 and 45 suggests a decoupling between both processes, as reported by Mutabaruka and colleagues (2007). This is probably because N immobilization is driven by both abiotic and biotic factors, whereas C mineralization depends on biotic controls.

Impact on microbial diversity

There have been several studies of microbial diversity fingerprints for bacterial communities in mesocosm experiments (Hewson *et al*., 2003; Hendrickx *et al*., 2005; Lejon *et al*., 2007), but none, in alpine soils, were carried out on the three main groups of microorganisms as we did here. We determined three distinct patterns, one for each microbial community. Previous studies showed that the bacterial SSCP patterns are specific for a given bacterial community (Godon *et al*., 1997; Mohr and Tebbe, 2006; Zinger *et al*., 2007a). Here, the bacteria profiles showed a high baseline suggesting a large number of rare phylotypes (Loisel *et al*., 2006), preventing the detection of minor changes. We found effects supported by bootstrapping for most treatments. However, because of the high baseline masking the community shifts (Fig. 3), the branch length remained very short between treatments (Fig. 2). Therefore, minor relevant changes in bacterial diversity cannot be detected and a more detailed study is needed to assess the tannin impact on bacterial communities.

For the crenarcheotes, freezing and tannin amendment resulted in a reduction in the number of peaks. Possibly, the convergence of both factors led to the disappearance of some crenarcheote phylotypes. However, the effects on nutrient cycling were likely to be negligible, as no decrease in population biomass or in C mineralization was detected.

Fungal communities, whose biomasses were found to increase during winter (Schadt *et al*., 2003), showed strong responses to all treatments. Tannin amendment associated with low temperature maintained a relatively high diversity whereas freezing temperatures alone led to a decrease in fungal richness. This result suggests that

some wintertime fungal strains may be able to benefit from the addition of tannins, as it has been already shown for bacterial communities (Chowdhury *et al*., 2004) or fungal populations (Mutabaruka *et al*., 2007). Previous studies on alpine meadows also suggested that a strong supply of allelochemical-rich litter in the fall may select wintertime populations able to grow on phenolic compounds (Lipson *et al*., 2002; Schmidt and Lipson, 2004). Unexpectedly, the phylotypes reacted differently in response to the addition of these compounds, depending on the thermic regime. This interaction may be related to the presence of psychrophilic fungi which were excluded at relatively high temperature. Another explanation is that the available labile C, which decreased at lower temperatures, created a selective pressure in favour of fungal strains which metabolize more resistant C substrates (Bradley *et al*., 2000).

Ecological implications

CO_2 efflux measurements showed that there were significant levels of microbial activities even well below 0°C (Brooks *et al*., 1998). In snow-covered ecosystems, litter decomposition occurs principally during the winter (Hobbie and Chapin, 1996) and recent studies indicate that winter microbial communities degrade phenolic compounds (vanillic and salicylic acids) better than summer microbial communities (Schmidt *et al*., 2000; Lipson *et al*., 2002). However, because of inconsistent snow cover during winter, dry meadows frequently experience very low temperatures (< –5°C) reducing soil microbial activity and litter decomposition rates (F. Baptist, unpubl. results, O'Lear and Seastedt, 1994). Furthermore, high concentrations of tannins in the fresh litter of *D. octopetala,* which is a dominant species in this ecosystem, potentially contribute to a decrease in N mineralization by complexing soil organic compounds (Northup *et al*., 1995; Hattenschwiler and Vitousek, 2000). Severe soil edapho-climatic conditions probably act by inhibiting microbial activity. However, we detected no toxic effects of compounds extracted from *D. octopetala* on microbial activity which indicates that plants producing phenolic compounds may select microbial populations able to use these compounds, or at least able to withstand them (Schmidt *et al*., 2000). Changes in phylogenetic composition coupled with higher C mineralization and protocatechuic acid contents showed that freezing temperatures selected psychrophilic fungi. These may be able to degrade *D. octopetala*'s tannins, and their activities are potentially initiated by a decrease in temperature. However, this particular effect of temperature remains unclear and could also be related to a decrease in labile C availability.

This study illustrates how soil and climatic conditions interact with soil microorganisms to enhance the metabolization of the tannins released by the plants which dominate alpine ecosystems. The degradation of recalcitrant compounds, during winter, produces a less recalcitrant litter which becomes available by the time plant growth starts. This limits nutrient immobilization thanks to a reduced litter C/N ratio (Schmidt and Lipson, 2004). Consequently, the microbial catabolism of these compounds during winter is of functional importance. A variation in snowfall might affect microbial functional diversity with cascading biogeochemical consequences on ecosystem processes and carbon sequestration. Nevertheless, further investigations remain necessary to identify the exact role of microorganisms in tannin catabolism and their vulnerability to climate change.

Experimental procedures

Field site

The study site was located in the Grand Galibier massif (French south-western Alps, 45°0.05′N, 06°0.38′E) on an east facing slope at 2520 m. The growing season lasts around 169 ± 6 days and the mean soil temperature is 7.7 ± 1.5°C in summer and –2.2 ± 1.7°C in winter. The mean soil temperature reaches very low values (< –5°C) during relatively long periods because of inconsistent snow cover. Dry meadow soils are classified as typical alpine rankers. The bedrock is calcareous shales. The dominant plant community in the field site consist mainly of *K. myosuroides* (Cyperaceae) and *D. octopetala* (Rosaceae). Fifteen soil cores were sampled in October 2005 using sterilized (ethyl alcohol 90°) PVC pipes (h = 10 cm, Ø = 10 cm) and tools, to avoid contamination. In the laboratory, the plants were separated from the soil cores which were covered with perforated plastic bags and stored at 0°C until the beginning of the experiment.

Experimental design

On day 0, three soil cores were destructively harvested and used as controls (Table 3), six soil cores were amended with 19 ml of a tannin solution (with a mean of 749 mg of C/core or 32.4 mg C g^{-1} soil C, tannin treatment, T) and six cores with sterilized water (water treatment, W) to reach similar gravimetric soil moisture contents (34.2 ± 1.7% and 34.1 ± 1.6% for cores amended with tannins and water respectively). Three W cores and three T cores were incubated at –6°C for 2 weeks (freeze treatment, F) and then at 0°C for four more weeks. The six remaining cores were kept at 0°C (stable temperature treatment, S) during the whole period. To limit temperature gradients inside the incubators, the soil cores were regularly rotated. At the end of the first period (day 15), half of each soil core (3 S/T, 3 S/W, 3 F/T, 3 F/W) was harvested for a first analysis (longitudinal section). To limit disturbance, the harvested soil was replaced by a sterile and closed bag full of sand. The remaining soil cores were placed back in the incubator for four more weeks at 0°C and then were harvested for final analysis.

Table 3. A. Soil characteristics of the cores. B. Initial parameters estimated on the three control soil cores.

(A) Soil characteristics	
Soil water content (%)	32.9 (3.7)
Bulk soil density on < 2 mm fraction (g cm^{-3})	0.24 (0.04)
Organic matter (%)	16.9 (4.3)
pH (H_2O)	5.1 (0.1)
pH (KCl)	4.1 (0.1)
Grain size analysis	
Clay (< 2 μm)	9.7 (0.5)
Silt (2–50 μm)	41.4 (1.0)
Sand (50–2000 μm)	48.6 (1.2)
(B) Initial parameters (T_0)	
Microbial biomass (mg C g^{-1} C)	170.6 (37.3)
Bacterial count (10^9 cells g^{-1} C)	1.45 (0.21)
Fungal biomass (μg ergosterol g^{-1} C)	130.2 (14.0)
Tannin (mg g^{-1} C)	0.30 (0.10)
NO_3^-(μg N g^{-1} soil)	0.10 (0.02)
NH_4^+ (μg N g^{-1} soil)	3.25 (0.10)
N_{org} (μg N g^{-1} soil)	914.2 (65.7)
Potential mineralization	
NH_4 production (μg N g^{-1} soil day^{-1})	12.1 (2.9)
N_{org} production (μg N g^{-1} soil day^{-1})	145.9 (40.9)

$n = 3 \pm$ standard error of the mean.

At each sampling time (day 0, day 15 and day 45), the soils were sieved (2 mm) and further analysed to determine the tannins and phenolic acid contents, microbial and fungal biomasses, bacterial counts, microbial diversity and nitrogen mineralization rates. The CO_2 efflux was measured between each harvest.

Soil characterization

Soil water content, pH_{H2O}, pH_{KCl}, bulk soil density and texture were determined following standard methods (Robertson *et al.*, 1999). The SOM content was determined by loss-on-ignition and the C mass was calculated by dividing SOM fraction by 1.72. In order to determine bulk soil density, the stones mass was determined and converted to stone volume using mean stone density of 2650 kg m^{-3} (Hillel, 1971).

Tannin extraction and phenolic analysis

Dryas octopetala leaves were collected at the end of July, and air-dried. Tannins were extracted from about 300 g of ground leaves, using liquid sequential extractions and a final purification on Sephadex LH-20 (Preston, 1999). The elemental composition of the dried final fraction was obtained by CHN analysis (C %: 62.4; N %: 0). The addition of tannins was performed with a solution of 15.85 g of purified tannins dissolved in 250 ml of distilled water. Proanthocyans (here after referred to tannins) were quantified in the soil extracts by spectrophotometry, after hydrolysis with butanol/HCl using the proanthocyanidin assay (Preston, 1999). The calibration curves were prepared with a previously purified proanthocyan fraction from *D. octopetala*. Phenolic acids were obtained (5 g FW) by a double ethanolic extraction (ethanol 70%) under reflux. Aliquots (20 μl) of the ethanolic solution filtered at 0.5 μm, were used for high-performance liquid chromatography (HPLC) analysis on a RP C18 μBondapak column (4.6 mm × 250 mm) monitored by a Waters 600 Controller with a UV detection at 260 nm (Waters 996 PDA). Phenolic acids were separated using a linear gradient from 0 to 20% of solvent B (acetonitrile) in solvent A (acetic acid 0.5% in distilled water) in 45 min, at 1.5 ml min^{-1}. Standards of common phenolic acids (including protocatechuic) were obtained from Sigma-Aldrich (L'Isle d'Abeau, France).

Nitrogen mineralization

Nitrogen was extracted from fresh soil samples with 2 M KCl. The soil extracts were analysed for ammonia (NH_4^+) and nitrate/nitrite (NO_3^-/NO_2^-) contents using an FS-IV autoanalyser (OI-Analytical, College Station, TX). The TDN content in the soil extracts was measured after oxidation with $K_2S_2O_8$ at 120°C. The DON contents in the soil extracts (μg N g^{-1} dw) were calculated as: [DON = TDN – ($N\text{-}NH_4^+$) + ($N\text{-}NO_3^-/NO_2^-$)]. The net nitrogen mineralization (MIN_{net}, μg N g^{-1} dw day^{-1}) between day 15 and day 45 was calculated as: $MIN_{net} = [[(-NH_4^+) + (N\text{-}NO_3^-/NO_2^-)]_{day45} - [(N\text{-}NH_4^+) + (N\text{-}NO_3^-/NO_2^-)]_{day15}]/dw/30$. MIN_{net} was not calculated between day 0 and day 15, because the day 15 did not originate from the same soil cores as those for day 0. The NMP was determined from subsamples, using anaerobic incubations (Waring and Bremner, 1964). This protocol allows comparisons of relative organic matter degradability in different soils. Under optimized conditions (dark, 7 day, 30°C, anaerobic) organic N in fresh soils was mineralized and accumulated as NH_4^+. The difference between the NH_4^+ in the fresh soil (t_1) and after the anaerobic incubation (t_2) gave the N mineralization potential: NMP (mg $N\text{-}NH_4$ g^{-1} dw day^{-1}) = [[($N\text{-}NH_4$) t_2 – ($N\text{-}NH_4$) t_1]/dw/7].

Soil CO_2 efflux

Throughout the experiment, CO_2 efflux measurements were conducted just after tannin amendment (day 0), one before temperature shift (day 15) and three times between days 15 and 45 on all soil cores. The cores were enclosed in a hermetic Plexiglas™ chamber equilibrated to 400 p.p.m. prior to measurements. The chamber was connected to a LiCor 6200 gas exchange systems (LiCor, Lincoln, NE, USA). Data recording lasted 3–5 min, depending on the signal fluctuations, and the soil temperature was monitored.

Microbial community analyses

Microbial biomass and ergosterol determination. Microbial carbon biomass was determined by the fumigation-extraction method (Jocteur Monrozier *et al.*, 1993; Martins *et al.*, 1997) adapted from Amato and Ladd (1988). Duplicated soil samples (10 g) were fumigated for 10 days with chloroform. Total organic nitrogen was extracted with 20 ml of 2 m KCl from both the non-fumigated and fumigated soil samples (T_0, T_{10}), microbial nitrogen biomass being determined from the difference between the two treatments. After reaction with ninhydrin, the absorbance (570 nm) of all samples was deter-

mined by spectrophotometry using leucin as standard. The microbial carbon biomass calculated using a conversion factor of 21 (Amato and Ladd, 1988; Martins *et al*., 1997). The soil ergosterol content was evaluated as an indirect estimate of the soil fungal biomass (Nylund and Wallander, 1992; Gors *et al*., 2007). Ergosterol was extracted from 5 g of soil (FW) with 30 ml of 99.6% ethanol by shaking for 30 min at 250 r.p.m. The soil solution was filtered and immediately submitted to HPLC under isocratic flow of 1.5 ml min^{-1} of MeOH, on a Lichrosorb RP18 column (250 × 4.6 mm, 5 μm). Calibration curves at 282 nm were recorded with standard ergosterol solution from Sigma-Aldrich (L'Isle d'Abeau, France).

Bacterial counts. The soil bacterial counting was conducted using the method described by Martins and colleagues (1997). Briefly, 10 g of soil (duplicated) was blended in 50 ml of sterile NaCl 0.9%. After flocculation of the soil particles, an aliquot of the soil suspension (1 ml) was collected and used to enumerate the bacteria after successive dilutions. One millilitre of the diluted suspension was filtered on 0.2 μm polycarbonate membrane filters (Millipore). Bacteria were then stained using a sterile solution (filtered at 0.2 μm) of 4′,6-diamidino-2-phenylindole (DAPI) and enumerated by direct counting with a motorized epifluorescent microscope (Axioscope, Zeiss) under UV excitation (Hg lamp) with a filter set for DAPI (365 nm) at 1000-fold magnification.

SSCP analysis of microbial diversity. DNA extraction and PCR: the protocols for fungal and prokaryotic signatures have already been described in Zinger and colleagues (2007a,b). Briefly, the soil DNA was amplified using microbial community-specific primers and submitted to capillary electrophoresis-SSCP (CE-SSCP). The 16S rRNA gene was used as the prokaryotic specific marker. The bacterial primers were W49 and W104-FAM (Zumstein *et al*., 2000; Duthoit *et al*., 2003) and the crenarcheaotal primers were 133FN6F-NED and 248R5P (Sliwinski and Goodman, 2004). Fungal ITS1 was amplified with the primers ITS5 and ITS2-HEX (White *et al*., 1990). The soil DNA extraction was performed with the Power Soil™ Extraction Kit (MO BIO Laboratories, Ozyme, St Quentin en Yvelines, France) using 250 mg (fresh weight) of soil per core sample, according to manufacturer's instructions. The DNA extracts from the same-condition cores were then pooled to limit the effects of soil spatial heterogeneity. The PCR reactions (25 μl) were set up as follows: 2.5 mM of $MgCl_2$, 1× of AmpliTaq Gold™ buffer, 20 g l^{-1} of bovine serum albumin, 0.1 mM of each dNTP, 0.26 μM of each primer, 2 U of DNA polymerase (Applied Biosystems, Courtaboeuf, France) and 1 μl of DNA (1–10 ng DNA). The PCR reaction was performed as follows: an initial step at 95°C (10 min), followed by 30 cycles at 95°C (30 s), 56°C (15 s) and 72°C (15 s), and final step at 72°C (7 min). The PCR products were visualized on a 1.5% agarose gel. Then, amplicons of each microbial community were then pooled for each sample to perform multiplex CE-SSCP. Capillary electrophoresis-SSCP: 1 μl of the PCR product was mixed with 10 μl formamide Hi-Di (Applied Biosystems, Courtaboeuf, France), 0.2 μl standard internal DNA molecular weight marker Genescan-400 HD ROX (Applied Biosystems, Courtaboeuf, France), and 0.5 μl NaOH (0.3 M). The sample mixtures were denatured at 95°C for 5 min and immediately cooled on ice before loading on the instrument. The non-denaturing polymer consisted of 5% CAP polymer, 10% glycerol and 3100 buffer. Capillary electrophoresis-SSCP was performed on an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems, Courtaboeuf, France) using a 36-cm-long capillary. The injection time and voltage were set to 22 s and 6 kV. Electrophoresis was performed for 35 min. The CE-SSCP profiles were normalized in order to control for differences in the total fluorescence intensity between profiles.

Statistical analysis. We tested for differences between the temperature and tannin amendment treatments using Mann–Whitney rank sum test ($P < 0.05$) (Statistica 5.0, Statsoft. (1995) Statistica 5.0 Software. Statsoft, Tulsa, USA). Paired differences between days 15 and 45 sampling were tested using the Wilcoxon signed rank test ($P < 0.05$). The normalized profiles of SSCP were analysed by Neighbour-Joining analysis based on a matrix of Edwards distances (Edwards, 1971). The robustness of the resulting tree was assessed using 1000 bootstraps. The data analysis was performed using the Ape package of the R software (RDevelopmentCoreTeam, 2006).

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Variations spatio-temporelles de la microflore des sols alpins

Résumé

Les micro-organismes jouent un rôle crucial dans les processus écosystémiques. L'étude de la distribution spatio-temporelle des communautés microbiennes est donc nécessaire, particulièrement dans un contexte de changements globaux. Les micro-organismes étant très diversifiés et majoritairement non cultivables, l'étude de leur diversité et des facteurs responsables de l'assemblage des communautés nécessite des outils adaptés.
Les écosystèmes alpins montrent de forts gradients mésotopographiques et de régimes d'enneigements. Ces gradients engendrent une hétérogénéité spatiale du couvert végétal et des processus écosystémiques à des échelles réduites. Les contrastes de l'étage alpin s'appliquent aussi dans le temps, ceux-ci étant soumis à des froids intenses en hiver. Ces écosystèmes sont donc un modèle de choix pour l'étude des patrons spatio-temporels de la microflore du sol.
Ce travail s'est d'abord concentré sur l'optimisation d'une technique d'empreinte moléculaire, la CE-SSCP, mais aussi d'outils statistiques pour l'analyse de séquences d'ADN.
Les communautés bactériennes, fongiques et crenarchaeotes du sol ont été suivies deux années, par CE-SSCP et clonage/séquençage, dans deux habitats contrastés par leurs régimes d'enneigements. Cette étude a ensuite été étendue à l'échelle du paysage, sous divers couverts végétaux.
Ce travail montre que l'assemblage des communautés microbiennes alpines varie au cours des saisons et que l'hiver constituent un fort évènement sélectif. Cette étude montre également que les communautés microbiennes sont spatialement distribuées en fonction des régimes d'enneigements et de la végétation. Les facteurs directement responsables de tels patrons sont discutés.

Mots clés : *micro-organismes, CE-SSCP, écologie des communautés, dynamiques saisonnières, régimes d'enneigements, biogéographie.*

Spatial and temporal variations of microbial communities in alpine tundra soils

Abstract

Microorganisms play a crucial role in ecosystem processes. Understanding the spatio-temporal distribution of microbial communities is thus a central issue, especially in a context of global changes.
Microorganisms are largely diverse, but given that the great part of them is still uncultured, the use of suitable tools is required to evaluate their huge diversity and the factors responsible for the community assembly.
Alpine ecosystems display strong mesotopographical and snow cover regime gradients. These environmental gradients create a strong spatial heterogeneity in plant cover and ecosystem processes at reduced scales. Alpine tundra are also submitted to strong temporal contrasts, due the very low temperatures occurring during winter. These ecosystems are thus well suited to study the dynamic and spatial patterns of soil microbial communities.
This work first focused on the improvement of a molecular fingerprint technique, CE-SSCP, but also on the development of statistical tools for the analysis of DNA sequences.
Soil bacterial, fungal and crenarchaeal communities were followed up over two years by using CE-SSCP and cloning/sequencing, in two habitats contrasted by their snow cover regimes. This study was then extended at the landscape scale, under different plant covers.
This work shows that microbial communities' assembly in alpine soils varies throughout seasons and that winter conditions constitute a strong selective event. This study also shows that microbial communities are spatially distributed according to snow cover regimes and plant cover. The factors directly involved in such patterns are discussed.

Keywords : *microorganisms, CE-SSCP, community ecology, seasonal dynamics, snow cover regimes, biogeography.*