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Maintenance of soil functioning following erosion of microbial diversity

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Summary

The paradigm that soil microbial communities, being very diverse, have high functional redundancy levels, so that erosion of microbial diversity is less important for ecosystem functioning than erosion of plant or animal diversity, is often taken for granted. However, this has only been demonstrated for decomposition/respiration functions, performed by a large proportion of the total microbial community, but not for specialized microbial groups. Here, we determined the impact of a decrease in soil microbial diversity on soil ecosystem processes using a removal approach, in which less abundant species were removed preferentially. This was achieved by inoculation of sterile soil microcosms with serial dilutions of a suspension obtained from the same non-sterile soil and subsequent incubation, to enable recovery of community size. The sensitivity to diversity erosion was evaluated for three microbial functional groups with known contrasting taxonomic diversities (ammonia oxidizers < denitrifiers < heterotrophs). Diversity erosion within each functional group was characterized using molecular fingerprinting techniques: ribosomal intergenic spacer analysis (RISA) for the eubacterial community, denaturing gradient gel electrophoresis (DGGE) analysis of *nirK* genes for denitrifiers, and DGGE analysis of 16S rRNA genes for betaproteobacterial ammonia oxidizers. In addition, we simulated the impact of the removal approach by dilution on the number of soil bacterial species remaining in the inoculum using values of abundance distribution of bacterial species reported in the literature. The reduction of the diversity of the functional groups observed from genetic fingerprints did not impair the associated functioning of these groups, i.e. carbon mineralization, denitrification and nitrification. This was remarkable, because the amplitude of diversity erosion generated by the dilution approach was huge (level of bacterial species loss was estimated to be around 99.99% for the highest dilution). Our results demonstrate that the vast diversity of the soil microbiota makes soil ecosystem functioning largely insensitive to biodiversity erosion even for functions performed by specialized groups.

Introduction

Unprecedented rates of species extinction (Chapin *et al*., 2000) resulting from anthropogenic disturbances have prompted extensive research on the impact of biodiversity losses on ecosystem functioning (Loreau *et al*., 2002). A fundamental question is whether ecosystems or functional groups with declining numbers of species can maintain process rates essential for ecosystem sustainability. During the last decade, several studies have provided evidence of the functional importance of biodiversity to ecosystem processes (e.g. Hooper and Vitousek, 1997; Symstad and Tilman, 2001; Wardle and Zackrisson, 2005). A recent synthesis (Loreau *et al*., 2002) stresses that most biodiversity-ecosystem functioning (B-EF) studies have been carried out on a small number of processes and systems, in particular primary production and nutrient retention in grasslands and, to a lesser extent, litter decomposition in soil and primary production in aquatic systems (McGrady *et al*., 1997; Naem and Li, 1997; Mikola and Setälä, 1998; Wardle, 2002).

Soil microbes possess vast phylogenetic and functional diversity and are key drivers of energy flow and nutrient cycling. Understanding the functional role of microbial diversity is therefore crucial for assessment of (i) the generic relevance of B-EF relationships (Loreau *et al*., 2002) and (ii) the impact of global change on terrestrial ecosystems. Many studies have reported the existence of correlation or lack of correlation between changes in soil microbial diversity and functioning in response to different treatments (e.g. Avrahami *et al*., 2003; Seghers *et al*., 2003; Webster *et al*., 2005; Patra *et al*., 2006). However,

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in these studies, impacts of changes in microbial diversity and environmental factors on functioning can be confounded. Studies directly addressing B-EF relationships for soil microorganisms have employed an assembly approach on a small number of symbiotic or litter-decomposing fungi (van der Heijden *et al*., 1998; Jonsson *et al*., 2001; Setälä and McLean, 2004). Others (Salonius, 1980; Degens, 1998; Griffiths *et al*., 2000; 2001; 2004) have used a removal approach. However, an important restriction of the latter studies was that they focused on a general and widespread function (decomposition), involving a wide range of substrates metabolized by microorganisms distributed throughout the prokaryotic kingdoms, thereby involving a large proportion of the total soil microbial community that likely possesses a high level of functional redundancy. There is no such study for soil ecosystem functions performed by more specialized, less diverse microbial functional groups. The generic value of the statement that soil microbial communities have high functional redundancy levels and that erosion of microbial diversity has little impact on ecosystem functioning beyond decomposition process has therefore never been critically tested and thus never demonstrated (Wolters *et al*., 2000; Bengtsson *et al*., 2002; Nannipieri *et al*., 2003).

The objective of this study was to quantify the impact of a decrease in soil microbial diversity on general but also specialized soil ecosystem functions using a removal approach in which less abundant species were removed first. Our study focused on the impact of biodiversity erosion within microbial functional groups with contrasting taxonomic diversities (i.e. ammonia oxidizers < denitrifiers < all bacterial heterotrophs) on the functioning of these groups. To address this issue, we established a range of microbial diversities by reducing richness through inoculation of sterile soil microcosms with serial dilutions of a suspension obtained from the same non-sterile soil.

Results

Abundances of bacteria within functional groups

The final abundances (after incubation for 19 weeks) of heterotrophic bacteria of sterile soils microcosms inoculated with dilutions of non-sterile soil (treatments named 10^{-1}–10^{-8}) were similar in all microcosms (Fig. 1). For denitrifying and ammonia oxidizing bacteria, similar final abundances were obtained among treatments 10^{-1}–10^{-6} and 10^{-1}–10^{-3}, respectively, and were lower in microcosms inoculated with higher dilutions (Fig. 1).

Diversity of each functional group

For treatments with similar abundances after the incubation for 19 weeks (Fig. 1), the richness index of each functional group decreased progressively from microcosms inoculated with low soil dilutions to those inoculated with high dilutions (Fig. 2). In microcosms inoculated with low dilutions, some differences in molecular fingerprints were observed between replicates, but fingerprints of communities inoculated with high dilutions varied more between replicates, even for the most intense bands of the fingerprints (Figs 3 and 4).

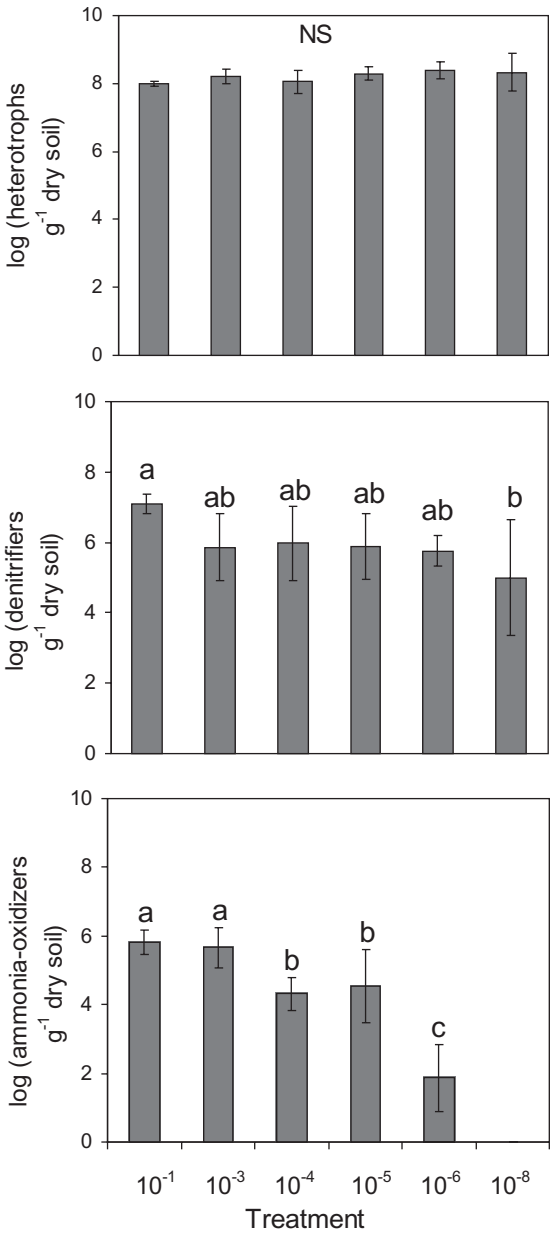


Fig. 1. Abundance of culturable heterotrophs, denitrifiers and ammonia oxidizers in sterile soil microcosms inoculated with a non-sterile soil suspension, giving final dilutions of 10^{-1}–10^{-8} of the non-sterile soil, after incubation for 19 weeks. Bars represent standard errors ($n = 4$). Values with different letters differ significantly ($P < 0.05$).

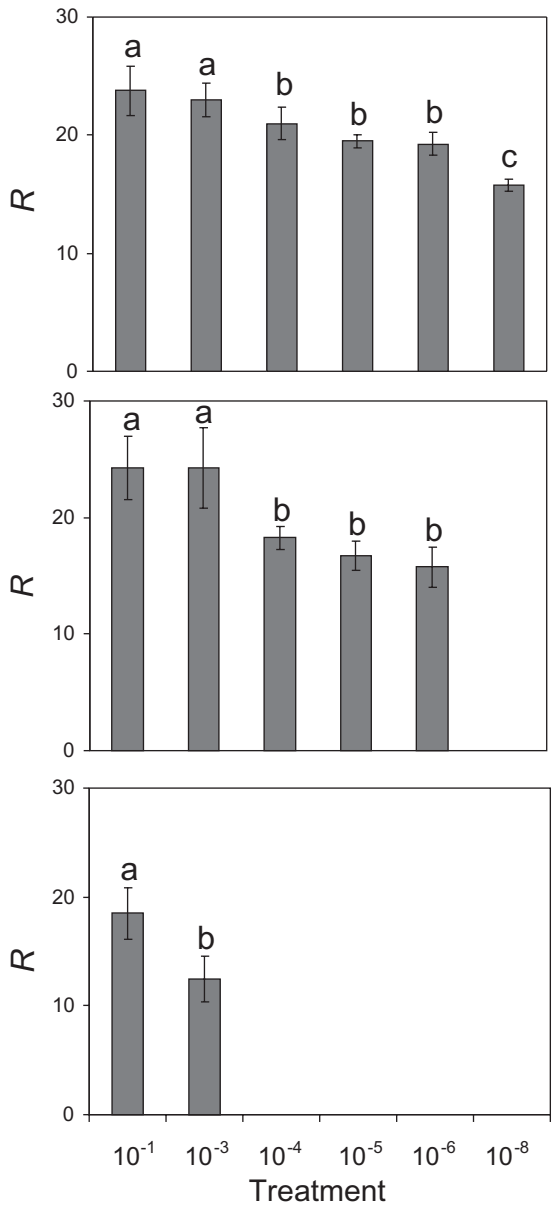


Fig. 2. Values of richness index (*R*) computed from fingerprints of (top) eubacteria, (middle) denitrifier and (bottom) ammonia oxidizer communities for treatments with similar functional group size. For details, see legend of Fig. 1.

Functioning of each functional group

Carbon mineralization did not differ significantly between treatments (10^{-1}–10^{-8}). Similarly, denitrification and nitrification did not vary significantly between treatments (10^{-1}–10^{-6} and 10^{-1}–10^{-3}, respectively) for which the abundance of the functional group was similar (Fig. 5). In addition, for the three activities, no significant correlations were obtained between activity level and treatment ($0.19 < P < 0.87$) or activity level and richness index ($0.21 < P < 0.93$) (data not shown).

Simulated effect of dilution on erosion of bacterial diversity

Simulation results (Fig. 6) show that, assuming that the abundance distribution of bacterial species reported by Gans and colleagues (2005) were valid for our soil, the level of species loss in the inoculum were around 21.8%, 86.5%, 97.7% and 99.99% for dilution treatments 10^{-3}, 10^{-4}, 10^{-5} and 10^{-8} respectively. Simulations also show that a pool of several hundreds of bacterial species remained in the inoculum for the 10^{-8} dilution treatment (inset in Fig. 6).

Discussion

The aim of our study was to characterize the impact of the erosion of soil microbial diversity on the functioning of

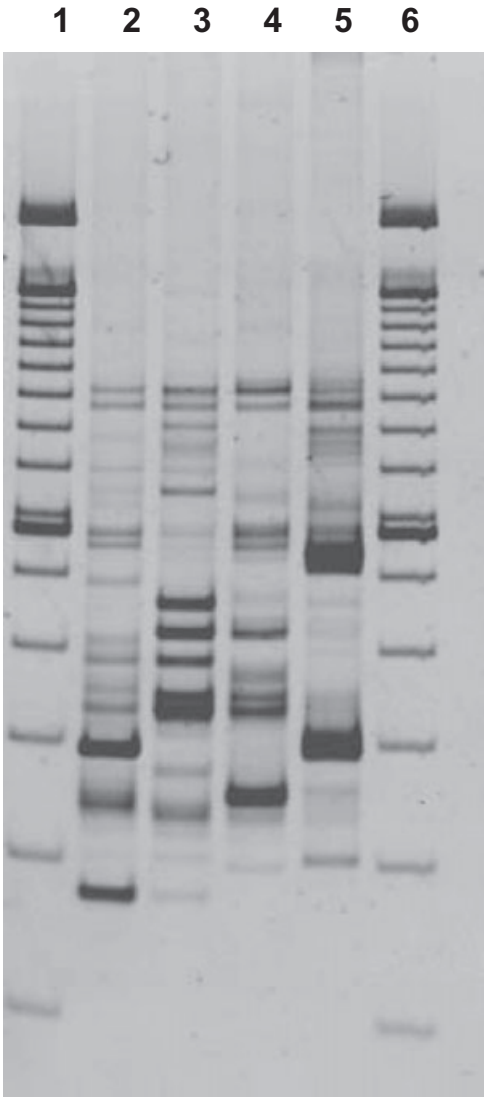


Fig. 3. Genetic profiles of bacterial community in four replicate soil microcosms of treatment 10^{-8} after incubation for 19 weeks. Lanes 1 and 6: 100 bp ladder; lanes 2–5: replicate ribosomal intergenic spacer analysis (RISA) profiles.

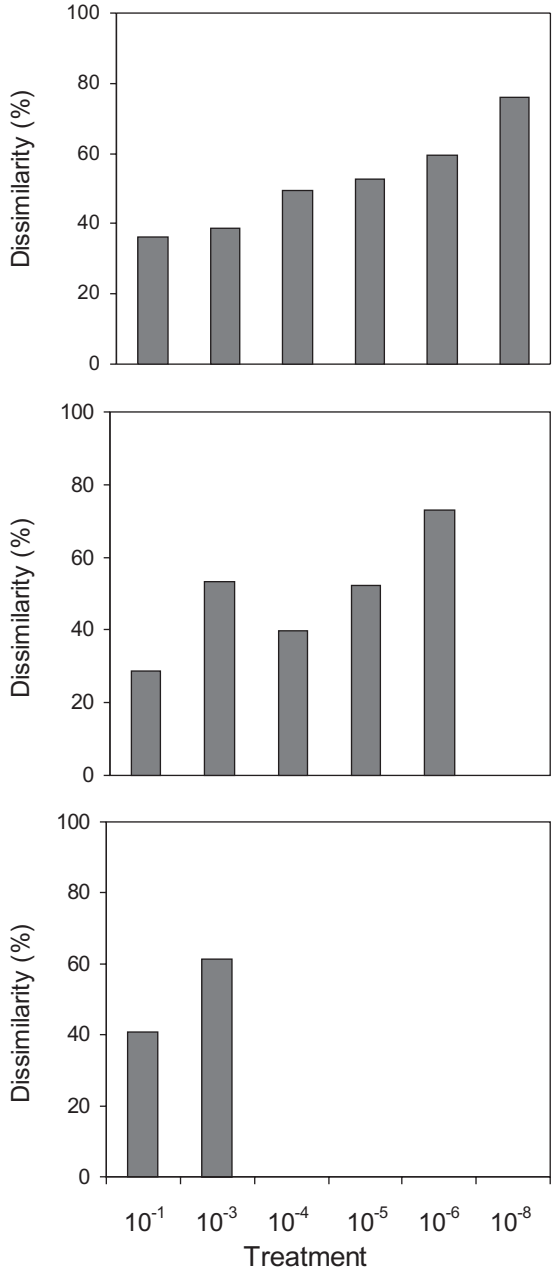


Fig. 4. Dissimilarity percentages of the genetic structures within four replicate soil microcosms as a function of dilution level (for each functional group, only treatments with similar group size are taken into account). Dissimilarity percentages were computed from fingerprints of (top) the eubacterial (middle) denitrifying and (bottom) ammonia oxidizing communities. For details, see legend of Fig. 1.

a soil ecosystem, focusing on both general and specialized soil functions (carbon mineralization, denitrification and nitrification). A range of microbial diversities was established by inoculation of sterile soil microcosms with serial dilutions of a suspension obtained from the same non-sterile soil and subsequent incubation, to enable recovery of community sizes. For each functional group, analyses of the relationship between diversity and function were restricted to those treatments with similar abundances. The fact that we obtained, after recolonization, lower abundances of denitrifiers and ammonia oxidizers in soil inoculated with high dilutions compared with soil inoculated with lower dilutions is likely because these organisms comprise decreasing proportions of the total community, and because ammonia oxidizers have low specific growth rates (Prosser, 1989).

Assessment of diversity within each functional group revealed that the experimental procedure we used produced a progressive decrease in richness for each functional group from microcosms inoculated with low soil

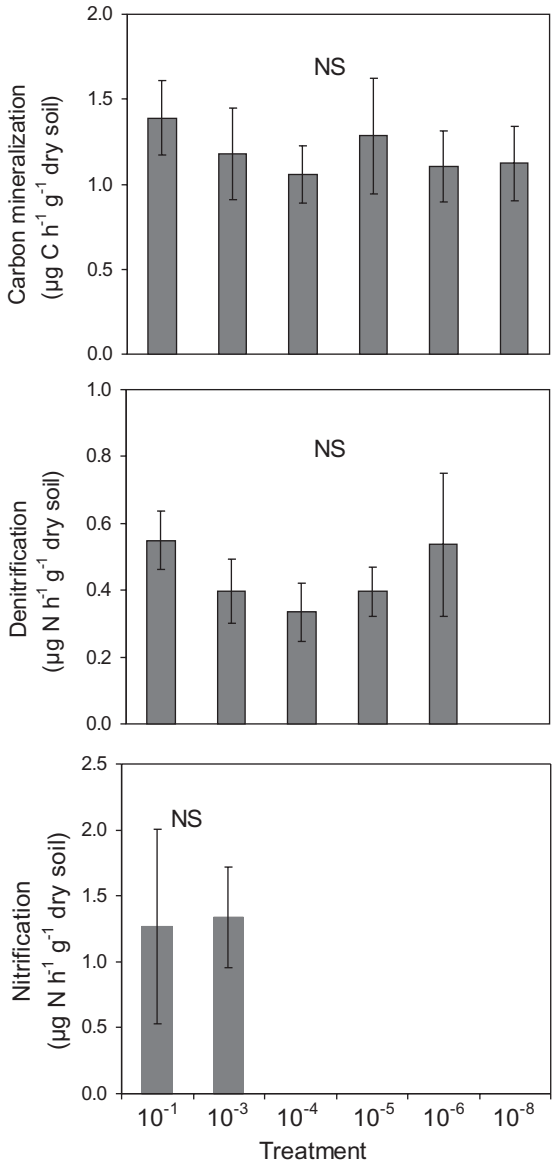
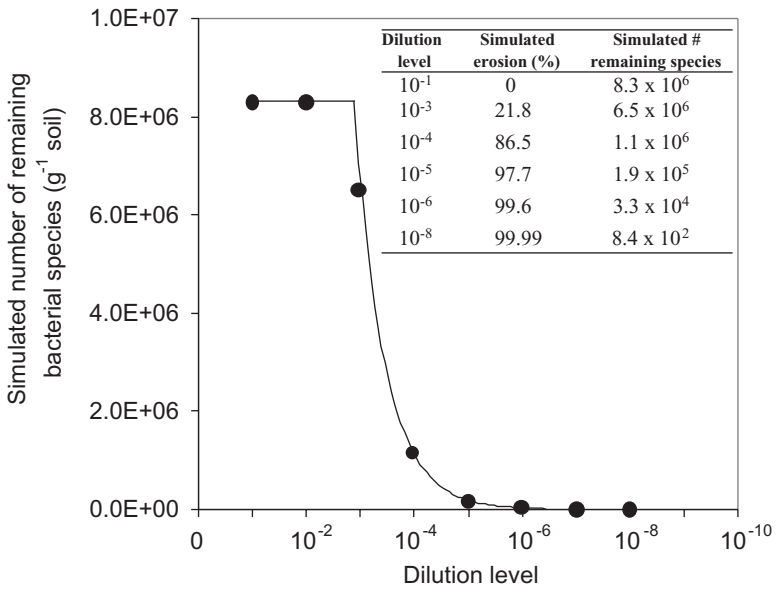


Fig. 5. (Top) Carbon mineralization, (middle) denitrifying enzyme activity, and (bottom) nitrifying enzyme activity for treatments with similar functional group size. For details, see legend of Fig. 1.



Dilution level	Simulated erosion (%)	Simulated # remaining species
10^{-1}	0	8.3×10^6
10^{-3}	21.8	6.5×10^6
10^{-4}	86.5	1.1×10^6
10^{-5}	97.7	1.9×10^5
10^{-6}	99.6	3.3×10^4
10^{-8}	99.99	8.4×10^2

Fig. 6. Simulated impact of a removal approach by suspension/dilution on the number of soil bacterial species remaining in the inoculum. Mathematical simulation was made using the frequency distribution of bacterial species reported by Gans and colleagues (2005) for a managed soil. Extinction of a species was assumed to occur when less than one member of that species remained in the inoculum at a given level of dilution. The inset provides, for each dilution level, the erosion level induced (% of species removed) and the simulated number of species remaining.

dilutions to those inoculated with high dilutions. This is consistent with the results obtained by Griffiths and colleagues (2000; 2001) for the soil eubacterial community using removal approaches. Molecular fingerprint approaches are adequate to evaluate relative changes in the diversity of microbial communities, but do not allow an accurate quantitative analysis of this diversity. Thus, the richness index computed in our study clearly shows that the erosion scenario used did induce a progressive decrease in microbial diversity, but did not allow us to quantify the amplitude of microbial diversity erosion. Methods available to precisely quantify microbial diversity such as DNA reassociation are highly time-consuming and currently prohibitive for application to large numbers of samples. Similarly, the survey size required for accurate analysis of microbial diversity is impractically large when using the cloning/sequencing approach, in particular if less abundant species must be accurately taken into account (Hughes, 1986). This is why we estimated the amplitude of diversity erosion generated by the dilution approach by mathematical simulation, using richness values and abundance frequency distributions of bacterial species reported for a soil (Gans *et al*., 2005) for which the value of bacterial community size was consistent with the total number of cultivable bacteria measured on our study soil. Our simulation results show that the amplitude of diversity erosion generated by the suspension/dilution approach was huge (level of species loss would be *c*. 99.99% for dilution treatment 10^{-8}). Because data on abundance distribution of species are available only for the total bacterial community, we could not evaluate the effect of the suspension/dilution approach for less diverse communities, i.e. denitrifiers and ammonia oxidizers.

In microcosms inoculated with low dilutions, differences in molecular fingerprints were observed between replicates. Given the good repeatability of the amplification/fingerprinting procedure used (i.e. the same profile was obtained when a given sample was run twice), this was likely due to the complexity of soil microbial communities that does not allow achievement of exact replication of community structure. As observed by Griffiths and colleagues (2001) for the soil eubacterial community, variation between replicates of fingerprints of communities inoculated with high dilutions was greater than those from low dilutions, due to stochastic effects associated with species removal and initial stages of colonization.

Our results show that, despite the progressive decrease in richness of eubacterial community, carbon mineralization was unaffected, which is consistent with results of Griffiths and colleagues (2001). In addition, our results show that decreasing the richness of denitrifying and ammonia-oxidizing communities did not affect their associated soil functions (denitrification and nitrification). Thus, significant decrease in richness in soil communities did not impair soil ecosystem function, highlighting the low sensitivity of the functioning of soil microbial communities to diversity erosion even for the most specialized communities. The variability observed in community structure between replicates corresponding to high dilutions indicates that our data cannot be explained by consistent selection for some species.

The maintenance of functioning of microbial functional groups does not necessarily suggest that all microbial species would play similar roles and that no functional complementarity exists among species. One hypothesis is that less abundant species, that are preferentially lost, are functionally less important. However, the high diversity

of soil microorganisms suggests that a more probable hypothesis is that the number of species remaining after diversity erosion was sufficiently high to allow maintenance of functioning. This is supported by our simulation of the dilution effect. Indeed, we simulated that several hundreds of bacterial species could remain in the inoculum for the highest dilution treatment (10^{-8}), which can provide significant scope for functional diversity.

More generally, our approach involving progressive removal of soil bacterial species according to their abundances is a realistic scenario of species loss. Indeed, Gans and colleagues (2005) showed that rare taxa that represented > 99.9% of the total number of bacterial taxa were purged following exposure to high metal concentration. In our approach, progressive removal of species implies (i) an initial loss of less abundant species due to dilution, and (ii) differences in regrowth abilities of and interactions between the remaining species during the recolonization phase. So, the final outcome of diversity erosion for soil ecosystem functioning results from the loss of organisms belonging to a given functional group but also from the collateral response of other organisms, which is itself of great interest for predicting the impact of soil biodiversity erosion (Diaz *et al*., 2003).

The three functional groups investigated represent different degrees of specialism. Carbon mineralization is carried out by the majority of bacterial heterotrophs; approximately 0.1–5% of cultured bacterial species in soil carry out denitrification (Tiedje, 1994); and soil ammonia oxidation is restricted to a single monophyletic group of betaproteobacterial ammonia oxidizers (Kowalchuk and Stephen, 2001). Although additional studies testing the effect of other scenarios of diversity loss are needed, the consistency of our results in the three functional groups studied indicates that high levels of functional redundancy are likely to be observed broadly for other functions and functional groups, and that biogeochemical cycles will be relatively unaffected by diversity loss if all functional groups are not eliminated and if their abundance can be recovered. This is an important characteristic for the sustainability of terrestrial ecosystems in the context of global change.

Experimental procedures

Soil microcosms

The 0–15 cm layer of a permanent grazed pasture soil located at Theix (45° North, 2° East) in France was collected (for soil characteristics, see Le Roux *et al*., 2003). Fresh soil was sieved (2 mm diameter mesh) and homogenized. Soil was sterilized by 100 kGy gamma irradiation from a ^{60}Co source (Ionisos, Dagneux, France) and stored at 4°C for 5 weeks before use. Soil sterility was checked by enumeration of heterotrophic bacteria by the most probable number (MPN) technique (Alexander, 1982).

Inocula for soil microcosms were prepared by homogenizing 125 g of soil (equivalent dry mass) in 250 ml of sterile demineralized water by grinding with a pestle and a mortar, followed by serial 10-fold dilution in demineralized water. Soil microcosms, consisting of 30 g of sterilized soil (equivalent dry mass) in flasks, were inoculated with 6 ml from each dilution giving inocula equivalent to 10^{-1}–10^{-8} g of non-sterile soil g^{-1} sterile soil. For each inoculum level, four replicates were established, and seven microcosms containing sterile soil were established as controls. Soil microcosms were incubated at 20°C and moisture content was maintained at 30%, equivalent to 70% water-holding capacity (WHC), by addition of sterile demineralized water. During 19 weeks of incubation, we surveyed microbial colonization (i.e. recovery of abundance of each functional group) in inoculated soil microcosms (Appendix S1). After incubation for 19 weeks, four replicate microcosms per dilution level were sampled for assessment of bacterial abundance, community structure and soil function.

Enumeration of bacteria within functional groups

Cultivable heterotrophic, denitrifying and ammonia oxidizing bacteria were enumerated by the MPN technique as described by Patra and colleagues (2005), except that 4 g of soil samples and fivefold serial dilutions were used. The lack of contamination during incubation was verified by enumeration of cultivable heterotrophic, denitrifying and ammonia oxidizing bacteria in control sterile soils.

Measurements of richness index for each functional group

DNA was extracted from 0.5 g of soil using the fast DNA SPIN Kit for soil (BIO 101 Systems; Qbiogene, Carlsbad, CA, USA). Community structure of eubacterial community was characterized by rRNA intergenic spacer (Ranjard *et al*., 2000), involving polymerase chain reaction (PCR) amplification of the intergenic spacer region between the sequence coding the large and the small ribosome subunits. Amplification was carried out using a thermocycler (T personal, Biometra, Göttingen, Germany) with an initial denaturation step of 5 min at 94°C, followed by 25 cycles of 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min, with terminal elongation at 72°C for 5 min. Polymerase chain reaction products were loaded on a 5% non-denaturing acrylamide gel (Euromedex, Mundolsheim, France) that was run for 15 h at 65 V in 1× TBE buffer (Bio-Rad, Ivry sur Seine, France).

Community structure of denitrifying bacteria was characterized by denaturing gradient gel electrophoresis (DGGE) analysis of *nirK* gene fragments amplified by nested PCR, using the primers of Liu and colleagues (2003) for the first PCR step and the same primers, modified by addition of a GC clamp to the 5′ end of the reverse primer, in the second PCR step. The final reagent concentrations for PCR were 1 μM primers, 200 μM of each dNTP, 1.75 U of Taq (Qbiogene, Carlsbad, USA), in 50 μl of 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, pH 9. Thermocycling conditions were: 5 min at 94°C followed by a touchdown between 72°C and 67°C (one cycle of 94°C for 30 s, 72°C for 1 min and 72°C for 1 min) and four subsequent cycles in

which the annealing temperature was decreased by 1°C/cycle to reach 68°C. Touchdown was followed by 25 cycles (30 s at 94°C, 1 min at 67°C and 1 min at 72°C) followed by 7 min extension at 72°C. Denaturing gradient gel electrophoresis analysis of PCR products employed the D-Code Universal Mutation Detection System (Bio-Rad) with a 6% polyacrylamide gel containing a gradient of 35–65% denaturant, 100% denaturing solution being defined as 7 M urea and 40% formamide. Gels were run for 4.5 h at 150 V in 1× TAE buffer at 60°C.

Ammonia oxidizing community structure was characterized by PCR-DGGE analysis of 16S rRNA genes with primary amplification by CTO189f and CTO654r primers (Kowalchuk *et al*., 1997), targeting the majority of betaproteobacterial ammonia oxidizers, and secondary amplification using bacterial 357f-GC and 518r primers (Muyzer *et al*., 1993). Amplification conditions and DGGE analysis are as presented by Freitag and Prosser (2003).

All gels were stained, photographed and analysed using BiocaptMW (Vilber-Lourmat, France) and Gel ComparII software (Applied Maths, Kortrijk, Belgium). For each functional group, richness (*R*) was estimated as the number of bands. For the three bacterial communities, the percentage of dissimilarity in genetic structures within replicates of each treatment was computed using PRIMER software (PRIMER-E, Plymouth, UK).

Soil ecosystem function

Carbon mineralization was determined by incubating 2 g of soil in closed plasma flasks for 7 h at 28°C. CO_2 concentration was determined in gas samples after 3, 5 and 7 h using a gas chromatograph (Agilent P200 Microcatharometer, USA).

Denitrifying and nitrifying enzyme activities were determined as production rates of N_2O (C_2H_2 was used to inhibit N_2O-reductase activity) and NO_3^-, respectively, over short time periods (8 h at 28°C). The methods used are described by Patra and colleagues (2005) except that 2 g of soil samples were used.

Statistical analyses

Analysis of variance was performed to test if the size, richness index and activity of each functional group differed between dilution treatments. Then, for each variable, Duncan's test was used to determine if means differed significantly. The significance of linear regressions between (i) activity of each functional group, and (ii) dilution treatments or richness of the community were also tested.

Mathematical simulation of erosion of bacterial diversity induced by dilution

We simulated the impact of the removal approach by suspension/dilution on the number of soil bacterial species remaining in the inoculum. Mathematical simulation was made using the total richness and frequency distribution curve reported by Gans and colleagues (2005) for a managed soil for which values of bacterial community size were consistent with the total number of cultivable bacteria measured on our native study soil. Gans and colleagues (2005) estimated the richness and frequency distribution of bacterial species on their soil by analysis of DNA reassociation kinetics accounting for uneven abundance of bacterial species. We mathematically simulated the impact of a dilution approach assuming that extinction of a species occurred when less than one member of that species remained in the inoculum at a given level of dilution. For each dilution level, we computed the erosion level induced (% of species removed) and the number of species remaining.

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Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Abundances of culturable heterotrophs during incubation time for 19 weeks (135 days) in sterile soil microcosms inoculated with non-sterile soil suspensions. For legibility, kinetics for treatments 10^{-1}, 10^{-3} and 10^{-6} only are presented. The abundance of culturable heterotrophs in the native, non-sterile soil is indicated for comparison.

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