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RESEARCH ARTICLE

Nutrient effects on the genetic and functional diversity of aquatic bacterial communities

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Abstract

Studies on the effects of biodiversity on ecosystem functioning have generally revealed a positive asymptotic relationship between biodiversity and single functions, suggesting species redundancy with respect to these functions. However, most research was performed on specific processes and did not consider ecosystem 'multifunctionality'. There is also little information on the relationship between genetic and functional diversity. To analyze this relationship, we performed a microcosm experiment on a complex lake assemblage of decomposers, in the presence of the green alga *Scenedesmus obliquus*, which acted as carbon source for decomposers. By manipulating nutrient enrichment and the N : P input ratio, we observed that the structures of particle-associated and free bacterial assemblages were highly predictable in response to stoichiometric constraints. For a given treatment, the taxonomic compositions of free and particle-associated bacterial communities appeared close to each other only when phosphorus was not depleted. A coinertia analysis revealed a clear coupling between the genetic diversity of the microbial community, assessed using PCR-denatured gradient gel electrophoresis, and its potential functional diversity, studied with Biolog Ecoplates. This suggests that an ecologically relevant fraction of bacterial communities is characterized by lower level of redundancy than frequently thought, highlighting the necessity of exploring further the role of biodiversity in multifunctionality within ecosystems.

Introduction

Over the past decade, considerable ecological research has focused on the effects of biodiversity on the functioning of ecosystems (for synthesis, see Hooper *et al.*, 2005). Studies generally showed a positive relationship between biodiversity and particular ecosystem functions, such as plant production (Schmid & Hector, 2004) or bacterial respiration (Bell *et al.*, 2005). This relationship was often asymptotic, suggesting species redundancy with respect to a single function. As underlined by Hector & Bagchi (2007), most research has been performed on specific processes and does not consider ecosystem 'multifunctionality'. The scientific community has reached a broad consensus on the role of biodiversity, in particular ecosystem properties. However, there is little information on the relationship between genetic and functional diversity. We can hypothesize that this relationship should be weak if only a small subgroup of generalists controls most processes within a community, but strong if each species only controls a small proportion of the overall processes.

In most experiments linking biodiversity and ecosystem functioning, species were randomly manipulated. Yet, species loss or persistence is not random, and some species combinations are very unlikely in nature (Leps, 2004). Taking into account assembly laws within communities might modify our view on the relationship between biodiversity and ecosystem functioning (Gross & Cardinale, 2005). When considering the overall system functioning instead of particular functions, the difference between random and natural assemblages might even be greater, depending on the covariance between processes. Schmid &

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Hector (2004) underlined that existing communities might lose species in many alternative orders. In such an uncertain context, the first generations of random biodiversity manipulations have provided highly valuable discoveries. Yet, contrasting community structures do exist in response to the environment. Thus, it also appears necessary to analyze the relationship between genetic and functional diversity in experiments where environmental factors constrain species composition. Aquatic experimental systems allow the manipulation of a large diversity of microorganisms and a vast array of environmental factors in simple and replicated environments, without suppressing the key features of ecosystem functioning. Thus, aquatic systems appear particularly appropriate for such approaches.

In the pelagic zone of aquatic ecosystems, primary production is essentially ensured by phytoplankton. Among several environmental parameters, nutrient availability strongly influences this production. Moreover, changes in nutrient load generate modifications of diversity, structure and functions at the levels of populations, communities and ecosystems, including genetic diversity, number of species and functional types, number of metabolic activities and food-web architecture (e.g. Hulot *et al*., 2000; Jeppesen *et al*., 2000; Paerl *et al*., 2003; Romo & Villena, 2005). Over the past decades, many studies have demonstrated the importance of heterotrophic bacteria both in nutrient cycling and transfer of part of the primary production towards upper trophic levels (Azam *et al*., 1983; Sherr *et al*., 1987). Primary producers and decomposers are the basis of the functioning of aquatic ecosystems. Because heterotrophic bacteria and autotrophic phytoplankton may not be limited by the same element, the increase in nutrient load may have distinct effects on their biomass (Danger *et al*., 2007a) and their diversity (Jacquet *et al*., 2002). Numerous studies have described the effects of nutrient enrichment on phytoplankton communities (e.g. De Costa *et al*., 1983; Gonzales, 2000). More recently, bacterial assemblages have also been shown to be strongly affected by modifications of the nutrient load (Dumestre *et al*., 2001; Sipura *et al*., 2005). In those studies, quantitative (increase in biomass) and qualitative (change in community structure) effects have been described. In a general way, the bacterial diversity has been discussed in terms of numbers of operational taxonomic units (OTU), often assessed with molecular methods. The genetic diversity of complex bacterial assemblages can be monitored with several cultivation-independent molecular fingerprinting methods, like the PCR-denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1997). The effects of nutrient load on the functional diversity of bacterial communities have been less studied (Lebaron *et al*., 2001). Few methods are available to assess this diversity. The Biolog EcoPlate^{TM} method provides indications of the potential functional diversity of bacterial communities via utilization patterns of 31 sole carbon sources (Garland & Mills, 1991). This method is particularly powerful for community comparisons (Preston-Mafham *et al*., 2002). Recently, several studies compared information on the genetic and functional diversity of bacterial communities, mostly in soil ecosystems (de Lipthay *et al*., 2004; Ritz *et al*., 2004; Maila *et al*., 2005; Krumins *et al*., 2006). Among the few studies performed on aquatic ecosystems, none tackled the issue of both the nutrient load and the N : P ratio impacts on these two aspects of the bacterial diversity.

Danger *et al*. (2007b) demonstrated that different phytoplankton-limiting factors can generate either competition for nutrients under phosphorus-limited conditions, commensalism under nitrogen-limited conditions, or mutualism for CO_2 under eutrophic nutrient-unlimited conditions between algae and bacteria. They used a system composed of two compartments: the very common green alga *Scenedesmus obliquus* (Turpin) Meyen and a complex community of decomposers derived from a natural lake community. The aim of the present work, based on the same experiment, is to determine how nutrient enrichment and N : P input-ratio affect both the genetic and the functional diversity of bacterial communities. Because specific bacterial communities can develop in the vicinity of algae or particles (Riemann *et al*., 2000; Pinhassi *et al*., 2004), the genetic diversity response to contrasted nutrient inputs was compared for free-living and for particle-associated bacteria. Genetic diversity was assessed using PCR-DGGE, and potential functional diversity was studied using the Biolog Ecoplates.

Materials and methods

Experimental setup

The algal model used is an axenic strain (CCAP 276/6a) of the green alga *S. obliquus* (Turpin) Meyen. A bacterial community from the mesotrophic Lake Créteil (France) was inoculated into the cultures. Lake water containing bacteria was filtered twice through sterile glass microfiber filters (Whatman GF/C, 1.2 μm) to limit contamination by other organisms. One milliliter of filtered water was added to each treatment. Posterior analyses using epifluorescence microscopy did not reveal any occurrence of pico-autotrophic organisms in the cultures. Cultures were carried out for 7 weeks under four different conditions, in triplicate: (NP) nutrient-rich (1000 μM N-$NaNO_3$ and 50 μM P-KH_2PO_4) standard COMBO medium (Kilham *et al*., 1998), (nP) nitrogen-depleted, (Np) phosphorus-depleted and (np) nitrogen–phosphorus-depleted medium. In the last three conditions, the concentration of the deficient

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nutrient in the medium was reduced to 1% of the concentration in the standard COMBO medium. The NP and np media had the same N : P ratio. The media were sterilized before being inoculated with *Scenedesmus* and bacteria. Microcosms were named according to their nutrient content (NP, nP, Np and np) and their replicate number (1, 2 and 3). Cultures were grown in 500-mL Erlenmeyer flasks containing 400 mL of culture medium in a climate-controlled room at 23 °C. Cultures were stirred, using a conventional magnetic stirring device, and a 12 : 12 h light : dark cycle was used under an intensity of $100\,\mu E\,m^2\,s^{-1}$. Initial concentrations were 10^4 cells mL^{-1} for algae and 1.64×10^4 cells mL^{-1} for bacteria. At the end of the experiment, 100 mL of each culture was successively passed onto 3-μm pore-size (polycarbonate) and 0.22-μm pore-size (cellulose acetate) filters to separate particle-associated and free bacterial communities. One hundred milliliters of axenic *S. obliquus* culture was also filtered onto a 3-μm pore-size filter. They were stored dry at − 80 °C and used later as a control for genetic analyses.

Counting of microorganisms and stoichiometric analysis of algae

Total bacterial densities were determined by counting the samples using epifluorescence microscopy (Leitz Dialux 22 equipped with a HBO 100 W lamp) after staining with 4′,6-diamidino-2-phenyl-indoldihydrochloride (DAPI, $0.4\,\mu g\,mL^{-1}$) for 15 min in the dark (excitation: 270–380 nm–emission: 410–580 nm) (Porter & Feig, 1980). Cells of *Scenedesmus* were counted at the end of the experiment in a Malassez counting chamber under a DIC light microscope (Leitz Diaplan, magnification × 100). Each algal culture in the steady state was filtered onto preweighed Whatman GF/A glass fiber filters (nominal cutoff 1 · 6 μm). These filters were then used to quantify the percentage of carbon and nitrogen contained in this organic matter using a CHN elementary analyzer (NA 1500 Series 2, Fisons, Manchester, UK). Organic phosphorus content was also determined after persulfate digestion, as already described in Danger *et al.* (2007b). Algal C : N : P ratios were expressed as molar ratios.

DNA extraction, PCR amplification and DGGE

DGGE is a classical fingerprinting method that helps separate amplified 16S fragments according to their sequence (Muyzer *et al*., 1997). Metagenomes of particle-associated bacterial communities were extracted from 3-μm pore-size filters (size fraction > 3 μm), and metagenomes of free bacterial communities were extracted from 0.22-μm pore-size filters (0.22 μm < size fraction < 3 μm). Genomic DNA of the axenic alga was also extracted and used later as a positive control in DGGE. The extractions were performed using the DNeasy Plant Mini Kit (Qiagen), the lysis buffer of the first step being directly applied on the filter. After extraction, DNA concentration was determined by fluorimetry after staining with the SYBR Green I (Sigma Aldrich, dilution 1 : 10 000). The measures were made on a Fluoroscan Ascent (Labsystem) (excitation: 485 nm, emission: 538 nm). The variable regions V3–V5 (500 bp) of 16S rRNA gene were amplified using primers 341-F et 907-R (Proligo, Primers Probes), specific of bacteria (Muyzer *et al*., 1997). A G–C clamp was added at the 3′ extremity of the primer 341-F in order to enhance the separation during the electrophoresis. The experimental protocol used is described in Lyautey *et al*. (2005). Amplified DNA was quantified on 1.65% agarose gel using the Molecular Mass Ruler (BioRad). The vertical electrophoresis was performed using a D-Code Universal Mutation Detection System (BioRad) as described by Muyzer *et al*. (1997). The vertical gradient of denaturant was 40–65% (where 100% denaturant corresponds to 7 M urea and 40% deionized formamide). DGGE was run for 18 h at 100 V and at 60 °C.

After electrophoresis, the gel was stained with SYBR Green I (Sigma Aldrich, dilution 1 : 5000) and visualized using UV transillumination. An image was captured using a CCD camera and the BIOCAPT software (Vilbert Lourmat). DGGE bands, corresponding to OTUs, were scored as present or absent from the gel analysis. Because of constraints linked to the gel size, only two of the three replicates were analyzed.

The analysis of the identity of bacterial species associated with particles was beyond the scope of the study. Consequently, we did not sequence DGGE bands, which were therefore not assigned to bacterial groups.

Potential functional diversity

Community-level physiological profiles were assessed using the Biolog EcoPlate^{TM} (Biolog Inc., Hayward, CA). The EcoPlate contains three replicated wells of 31 carbon sources and three negative controls with no substrate. Metabolism of the carbon source results in the respiration-dependent reduction of a tetrazolium dye contained in each well in formazan, which induces the formation of a purple coloration and increases the OD of the solution. The Biolog study was performed at the end of the experiment. The plates were inoculated with 150 μL of culture sample filtered through a polycarbonate filter (3-μm pore size) in order to eliminate algae and other particulate organic matter. As algal respiration and bacterial activity on culture particulate substrates would have biased the estimation of bacterial metabolism, only the potential functional diversity of free bacteria was assessable. For one plate, the three replicates of one condition were used. The plates were incubated at 23 °C in the dark. The $OD_{590\,nm}$ was measured every 24 h for 10

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days, using a multiwell plate reader (Spectro Max plus 384, Molecular Device). Values of the respective control well were subtracted. Negative values that occasionally resulted were set to zero. We used the net absorbance at 120 h for the analyses. The substrates were assigned to chemical guilds: polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds (Choi & Dobbs, 1999). Analyses were conducted on the substrates and on the guilds. For this purpose, guilds were equally weighted to the largest one (carbohydrates). The sum of the OD of the substrates belonging to each guild was multiplied by a correcting factor, the number of substrates in the largest guild divided by the number of substrates of each guild. To compare the guild repartition of the bacterial community activity, the data on each treatment obtained as described above were divided by their sum. Then, all the values were ranged between 0 and 1 and differences in guild size were taken into account. The sample richness of metabolic functions was determined by the number of substrates whose OD was > 0.25 (Garland, 1996).

Statistical treatment of data

For genetic diversity analyses, bands of the two size fractions of each microcosm (> 3 and $< 3\,\mu m$) were pooled. Principal components analysis (PCA) (covariance matrix) was performed using the ADE 4 program for Windows (Thioulouse *et al.*, 1997). Plots were grouped according to the nutritive conditions. Two-by-two contingency tables were also established for each replicate in order to test the independence of the occurrence of each DGGE band as free or attached OTU. In the same way, we tested the independence between the occurrences of the different OTUs in the two replicates of a similar treatment, and in two different treatments. For each comparison, we used Fisher's exact test. This test constitutes the appropriate statistic for the analysis of categorical data where sample sizes are small or when data are rare. The probability values for the same or a stronger association are given in the result section.

For potential functional diversity, PCA was performed on both substrates and guild data. According to Ramette (2007), PCoA might better deal with the problem of the presence of many double zeros in data sets than PCA. However, with this analysis, the interpretation of variable contribution may be more difficult. We verified that cluster analyses performed on untransformed data or on the first axes of PCA analyses gave very similar results. This indicates that the potential problem of double zero in data did not modify the interpretation of the observed patterns and that our PCA results were robust.

A coinertia analysis (CoA) was performed (ADE 4 program) on the data from the 0.22-μm pore-size filters. CoA performs simultaneous analysis of two tables of data and thus allows the study of their relationship (for more details on the statistical method, see Doledec & Chessel, 1994). Here, the first matrix contains net absorbance data for the Biolog substrates and the second encodes (1 or 0) presence/absence of bacterial OTUs. Both matrices are first analyzed using PCA. CoA displays the maximum covariance between the two new sets of coordinates. The occurrence of a significant costructure between the two matrices was checked using a Monte-Carlo permutation test.

To compare organism densities, genetic diversities and functional diversities between nutrient treatments, ANOVAS were performed with cellular density, the presence/absence and net absorbance data (absorbance data corrected for controls) with the STATVIEW program for Windows (version 4.55). When necessary, logarithmic transformation of the data was carried out in order to normalize the residuals. *Post hoc* tests used Scheffé's procedure. For all statistical ANOVAS, significance was inferred at $P \leq 0.05$.

Results

Algal and bacterial abundance and elemental composition of algae

At the end of the experiment, algal density differed significantly between the rich medium and poor media ($P < 0.05$; Fig. 1a). The final bacterial density depended significantly on the nutritive conditions ($P < 0.0001$; Fig. 1b). It was maximal in NP microcosms (3.1×10^7 cells mL^{-1}) compared with nutrient-deficient microcosms (between 0.71×10^7 and 1.4×10^7 cells mL^{-1}). The ratio between bacterial density and algal density was significantly higher in the three nutrient-deficient media ($P < 0.05$).

The algal chemical compositions in the four treatments followed clear patterns according to the algal-limiting factor (see Danger *et al.*, 2007a for limiting factor analysis). The C : N : P molar ratios were 112 : 14 : 1 under CO_2-limited conditions (NP), 40 : 2 : 1 under nitrogen-limited conditions (nP), 1125 : 77 : 1 under phosphorus-limited conditions (Np) and 985 : 33 : 1 in nitrogen–phosphorus-limited (np) microcosms.

Genetic diversity of free and particle-associated bacterial communities

The PCR-DGGE allowed visualization of 87 OTUs (different band positions, Fig. 2). Some OTUs were specific to a nutritive condition while others overlapped between treatments (Table 1). Only two bands were present in almost all the microcosms. For pooled presence/absence data (both size fractions for each sample), the first two principal components of the PCA explained 50.1% of the variance (Fig. 3). Communities from each nutritive condition were

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Fig. 1. (a) Mean algal density at the end of the experiment. (b) Mean bacterial density at the end of the experiment. NP, standard COMBO medium; nP, nitrogen-deficient medium; Np, phosphorus-deficient medium; np, nitrogen- and phosphorus-deficient medium. The letters indicate statistically homogeneous groups. Vertical bars represent SEs.

Fig. 2. DGGE profile of the bacterial communities and corresponding number of bands. Communities are designed by their nutritive condition (see Fig. 1), their replicate number and the size fraction (a, $>3\,\mu m$; b, $<3\,\mu m$).

very clearly separated. Their replicates were very close, except the for np microcosms, which appeared more distant. This was confirmed using Fisher's exact test, which indicated highly significant associations between the replicates of the NP, nP and Np treatments ($P \leq 0.00001$), but no association between the two np microcosms ($P = 0.22$).

Table 1. Summary of the DGGE data

		NP	nP	Np	np
Number of bands (>3– $<3\,\mu m$)		31–26	24–25	32–32	36–44
Bands present in both size fraction		23	17	17	19
Number of common bands between treatments	NP	**34**			
	nP	17	**32**		
	Np	17	19	**47**	
	np	23	22	34	**61**

To obtain this table the DGGE profile of the replicates were pooled. The diagonals represent the total number of bands for each treatment (in bold).

Fig. 3. PCA of the presence/absence DGGE data, the two pore-size data being pooled. Axis 1 and 2 explain, respectively, 28.6% and 21.5% of the variance. Plots are grouped according to their nutritive condition (see Fig. 1) and their replicate number. Circles represent the gravity center of the plots for each group.

Fisher's exact tests revealed strong association between free and particle-associated bacterial communities in the two NP ($P < 0.000001$) and in the two nP ($P < 0.000001$ and $P < 0.001$) microcosms, but not in the two Np ($P > 0.89$ and $P > 0.95$) and in the two np ($P > 0.84$ and $P > 0.93$) flasks. In order to verify the general role of nutrient supply in the predominant phylotypes of the two size fractions, we compared the percentage of bands belonging simultaneously to the two size categories according to nitrogen and phosphorus supply. The percentage of bands belonging simultaneously to the two fractions was smaller under Np ($21.6 \pm 0.6\%$) than under the phosphorus-rich ($57.2 \pm 8.1\%$) conditions ($P = 0.011$). This result confirmed Fisher's exact test results suggesting more divergent free and particle-associated bacterial communities in the Np media.

There was no significant difference between nutritive conditions for the richness when considering separately free and particle-associated bacterial communities. Moreover, the number of bands did not differ significantly between

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the two fractions ($P = 0.56$), the mean richness being 21.4 ± 4.0 OTUs (mean ± SE).

The total number of OTUs per microcosm varied between 23 and 47, with a mean richness equal to 31.6 ± 8.1 (mean ± SE). This number of OTUs was significantly greater ($P = 0.022$) under low phosphorus load ($N = 38.0 \pm 6.4$ OTUs) than under high phosphorus conditions ($N = 25.2 \pm 2.2$ OTUs). We neither observed any significant nitrogen effect ($P = 0.29$) nor any phosphorus × nitrogen interaction effect ($P = 0.84$). Microcosm np1 appeared as an outlier with 47 OTUs, which was clearly higher than the mean number of DGGE bands observed in the other Np microcosms load ($N = 35.0 \pm 1.5$ OTUs). An ANOVA performed without this outlier confirmed the phosphorus effect ($P = 0.013$).

As a conclusion, the taxonomic compositions revealed using the PCR-DGGE were strongly dependent on the nutritive conditions. For a given treatment, the taxonomic compositions of free and particle-associated bacterial communities appeared close to each other only when phosphorus was not depleted. Under such Np conditions, the observed number of OTUs was also greater.

Potential functional diversity of free bacterial communities

Substrate utilization in the Biolog Ecoplates started later for np flasks (48 h of incubation) than for the other treatments. Net absorbance data at 120 h were analyzed using the PCA (Fig. 4). The first two principal components explained 75.3% and 9.8% of the variance, respectively. Three groups of microcosms clearly segregated: nP microcosms, Np microcosms and NP–np microcosms. The right half-plane (NP and np microcosms) was linked to the rather high utilization of the polymer Tween 80 and to the low utilization of most other substrates (Fig. 4b). Np and nP microcosms were well separated on the second half-plane. The superior quadrant (Np) corresponded to a high utilization of L-arginine, L-asparagine and D-mannitol and to a low utilization of polymers. The inferior one (nP) corresponded to higher utilization of galacturonic acid, pyruvic acid and polymers. Microcosm np1 appeared as an outlier in the PCA (Fig. 4).

The guild categorization of the community activity helped to identify some particularities of the community activity (Fig. 5). The PCA revealed a link between nP and Np communities, whose activity concerned mainly amines, amino acids and carbohydrates (Fig. 5a). Polymers were less catabolized by bacterial communities in the Np microcosms than in the nP ones. NP and np communities were characterized by a high utilization of polymers and phenolic compounds, respectively. There was no significant effect of the nutritive condition on the richness (number of metabolized substrates).

CoA

The CoA revealed a costructure between the genetic diversity data (size fraction $< 3\,\mu m$) and the functional diversity data for the replicates 1 and 2 ($r^2 = 0.46$, $P < 0.01$). The canonical graph demonstrated the correlation between the two data tables, the two coordinate systems being superposed in the coinertia plane (Fig. 6). This result remained the same when the np1 microcosm (outlier in the two diversity analyses) was excluded. The common structure between the two tables corresponded to a clear discrimination between NP, nP and Np communities. The np communities appeared close to the Np communities.

Discussion

Coexistence between algae and bacteria

Herein, we used a simplified system in microcosms to study the effects of nutrient load on the genetic and functional diversities of bacterial communities associated with an algal species. Bacteria and algae did coexist throughout this 7-week experiment. Although bacteria competed with algae

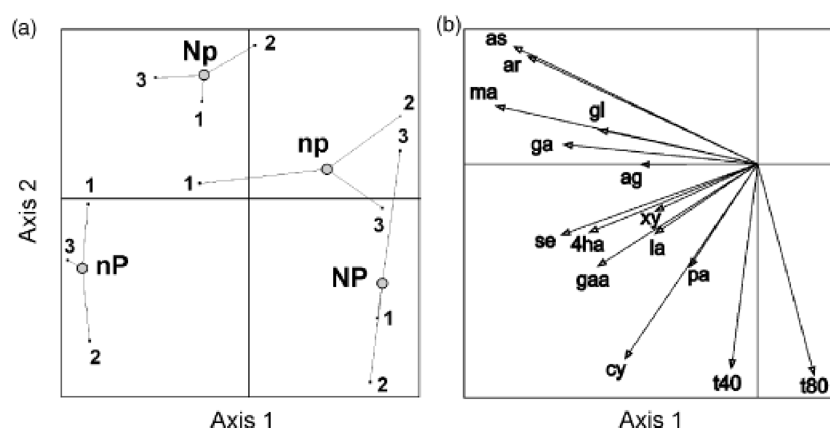

Fig. 4. (a) PCA of the Biolog data (net absorbance at 120 h). Axis 1 and 2 explain, respectively, 75.3% and 9.8% of the variance (see Figs 1 and 2 for symbols). (b) Factorial map of the Biolog substrates. Only the substrates that are more correlated to the axis are represented (4ha, 4-hydroxybenzoic acid; ag, *N*-acetyl-D-glucosamine; ar, L-arginine; as, L-asparagine; cy, α-cyclodextrine; ga, D-glucosaminic acid; gaa, galacturonic acid; gl, D-galactonic-γ-lactone acid; la, α-D-lactose; ma, D-mannitol; pa, pyruvic acid; se, L-serine; t40, Tween 40; t80, Tween 80; xy, D-xylose).

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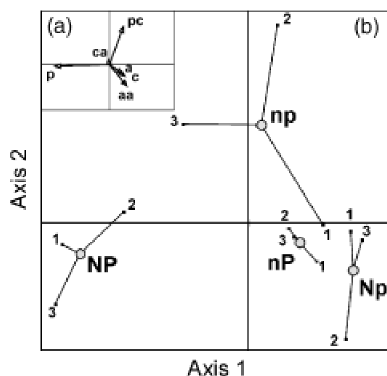

Fig. 5. (a) Factorial map of the Biolog guilds (a, amines; aa, amino acids; c, carbohydrates; ca, carboxylic acids; p, polymers; pc, phenolic compounds). (b) PCA of the Biolog data (guild repartition of the bacterial activity). Axis 1 and 2 explain, respectively, 59.6% and 29.7% of the variance (see Figs 1 and 2 for symbols).

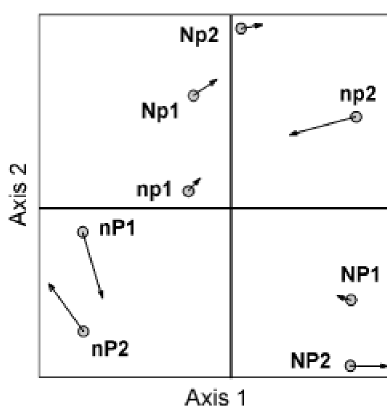

Fig. 6. Distribution of the communities on the first coinertia plane. Each arrow positions a community by its potential functional diversity (arrow start and circle) and its genetic diversity (arrow end). Communities are designed by their nutritive condition (see Fig. 1) and their replicate number. For the genetic diversity, analysis was only performed on the data from the 0.22-μm pore-size filters.

for nutrients, they probably could not exclude them because of their dependence on the carbon fixed by the primary producers (Daufresne & Loreau, 2001; Danger *et al*., 2007b). As expected from previous studies (Lebaron *et al*., 1999; Gonzales, 2000; Sipura *et al*., 2005), both algal and bacterial abundances increased with a simultaneous increase in nitrogen and phosphorus. The increase in density was more marked for algae than for bacteria as revealed by the lower bacteria : algae ratio in NP medium. This tendency has already been observed in other studies (see Cotner & Biddanda, 2002; Danger *et al*., 2007b), probably in relation to the lower DOC exudation rate by algae.

Nutrient status and number of OTUs

In this experiment, there was no visible effect of the nutritive conditions on the richness (number of OTUs) of the communities when considering free and particle-associated bacteria separately, despite a clear effect on community structure. Conversely, we observed a significant increase in OTUs in the Np microcosms on merging the two size fractions. This result is in accordance with the literature survey of Mittelbach *et al.* (2001). They showed that in most studies that have manipulated productivity, species richness was greater in low nutrient addition treatments than in high nutrient addition treatments. This decline has generally been interpreted as being linked to the dominance of a few previously nutrient-limited species able to outcompete other species. Note, however, that the absolute number of DGGE bands is probably a poor estimator of community richness (Muyzer & Smalla, 1998; Lindström *et al*., 2007). DGGE fingerprinting provides information on the dominant, and therefore on the competitively successful OTUs. As a general rule, each detected OTU represents $> 1\%$ of the community. Thus, we cannot draw any robust inference on the importance of nutrient load on bacteria taxonomic richness. Yet, we can draw two strong conclusions. First, nutrient status strongly affected the structure of bacterial communities within a given size class, in accordance with recent results from mesocosm experiments (Sipura *et al*., 2005). Second, a clear differentiation occurred between the structures of free and particle-associated bacterial communities under the Np conditions, while this did not occur between these two assemblages in the phosphorus-rich cultures.

Nutrients and bacterial genetic structure

In our study, there was a strong differentiation of the communities in terms of genetic diversity, depending on the nutritive conditions. PCA analysis indicated that Np and np communities were relatively close. This suggests that phosphorus status is important for determining the genetic structure of a bacterial community, in accordance with its key role in the physiology and ecology of bacteria (Vadstein, 2000). Moreover, Np and np communities clearly differed from nP communities. This indicates that the imbalance between nutrients probably plays a determinant role in the genetic structure of the bacterial community. These genetic differences are probably linked to the functional characteristics of bacteria, but might also partly be dependent on stoichiometric constraints, the chemical composition of bacterial communities being quite variable (Makino & Cotner, 2004) despite a relative homeostasis of specific taxa (Makino *et al*., 2003). The third class of genetically determined OTUs belongs to the NP media. Note that there is a good agreement between the results obtained on genetic diversity and the results obtained on the same experiment by Danger *et al.* (2007b), demonstrating that the interaction between bacteria and *Scenedesmus* had switched from net

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competition for nutrients under Np conditions, to commensalism under nP conditions and finally to mutualism for CO_2 under eutrophic (NP) conditions. This strongly suggests that the interaction between primary producers and decomposers played a key role in structuring bacterial communities. This interaction is not only linked to the availability of nitrogen and phosphorus but also to the production of dissolved organic carbon by algae (see Danger *et al*., 2007a, b). This agrees with Øvreås *et al*.'s (2003) results revealing moderate changes of bacterial community structure when mineral nutrients were added alone, but strong changes when manipulating inorganic nutrients and glucose together. Note also that we did not consider viruses in our experiment. Even if there are little data on the interactions among viruses, prokaryotes diversity and nutrient availability, recent studies have shown that resource availability can play a significant role in virus abundance and activity (Pradeep Ram & Sime-Ngando, 2008). Nutrient-mediated effects on viruses could have played a nonnegligible role in the determination of dominant OTUs in our experimental treatments. These interactive effects of nutrients, prokaryotes and consumers of prokaryotes certainly have important ecological implications for the functioning of lakes and will require further specific studies.

Particle-associated vs. free bacteria

The bacteria associated with algae can form very specific communities (Pinhassi *et al*., 2004). In the present study, differences between free and particle-associated bacterial communities tended to be abolished by a high phosphorus load while Np conditions induced clearly distinct communities. The eventuality of a bias due to retention of a large fraction of free particles onto 3-μm pore-size filters, which would have increased similarity between DGGE profiles in the two phosphorus-rich treatments, is very unlikely as the percentages of bands specific to free and particle-associated bacterial communities were similar and independent of phosphorus. If an important fraction of free bacteria had been retained onto filters, their predominant phylotypes would have also been important members of the DGGE profiles of attached communities, and only a low percentage of bands would have been found solely as free bacteria. The mean C : P molar ratio of particles was 1417 ± 216 in the Np media and 69 ± 8 in the phosphorus-rich one ($P < 0.0001$). This suggests that the differentiation between free and particle-associated bacteria may be partially controlled by nutrient availability and the quality of particulate organic matter. Riemann & Winding (2001) have proposed that in the presence of freshly formed, high-quality, phytoplankton detritus, there might be an extensive exchange of cells between heavily colonized particles, serving as 'baby machines' assimilate (Jacobsen & Azam, 1984), and the free-living phase. This would result in a significant overlap in the phylotypes of the two assemblages. Particle-associated bacteria are often more active, and produce higher specific exoenzyme concentrations than free-living ones on a cellular basis (Anesio *et al*., 2003). However, the activities of attached bacteria release more DOC than they assimilate (Jacobsen & Azam, 1984). Thus, free bacteria should also have benefited from labile DOC in our phosphorus-rich microcosms. On the contrary, bacterial specificity might be greater on the poor-quality particles occurring under the Np conditions. Such particles might constitute a very selective environment, favoring colonization by specialized bacterial communities with high ability to degrade more refractory organic components (Lemarchand *et al*., 2006). Moreover, the bacterial growth rate is probably low under such Np conditions, and particles should not play an important role as 'baby machines' and DOC providers for free bacteria: a weak activity of these free bacteria is expected. Thus, the difference between particle-associated and free bacteria should be greater in Np conditions. Consistently, Lemarchand *et al*. (2006) observed greater differences between the two bacterial communities in the oligo-mesotrophic Lake Pavin than in the eutrophic Lake Aydat. Other mechanisms might increase discrepancies between particle-associated and free bacterial communities. Long & Azam (2001) observed that a remarkably large fraction of marine bacteria species exhibited antagonistic properties against other pelagic bacteria. Their results also suggest that particle-attached bacteria are more likely to produce inhibitory compounds than their free-living counterparts, and that inhibition is greater on free bacteria. Thus, the relative importance of activity from the attached fraction should increase under nutrient-depleted conditions, even if free bacteria remain numerically dominant (Bidle & Fletcher, 1995; Lemarchand *et al*., 2006). The finding of such a context-dependent structure might help to reconcile contradictory results indicating either divergence (Bidle & Fletcher, 1995; Lemarchand *et al*., 2006) or significant overlap (Riemann & Winding, 2001) between free and particle-associated bacterial communities.

Sole-carbon-source utilization patterns

The utilization patterns of the 31 substrates of the Biolog EcoPlates^{TM} by the free bacterial communities confirmed the role of available resources in microbial functional capacities in the different treatments. When using ordination, three groups of microcosms clearly appeared: nP microcosms (algae characterized by low C : P, high C : N and very low N : P ratios), Np microcosms (high C : P, low C : N and high N : P ratios) and NP–np microcosms (either low or high C : P and C : N ratios but intermediate N : P ratios of algae). In accordance with the stoichiometric

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composition of particulate organic matter, nitrogen-rich amino acids such as L-arginine and L-asparagine were preferentially used in the Np microcosms. This is also in agreement with the results of Nagao & Miyazaki (2002) indicating that *Scenedesmus* cultivated under nitrogen-replete conditions release appreciable quantities of dissolved organic nitrogen, probably as amino acids or compounds of higher molecular weight. Carbohydrates also constituted a preferential substrate for bacteria in Np microcosms. Such molecules are frequently released by phytoplankton under conditions of high light and low phosphorus supply, typical of these microcosms (see Danger *et al*., 2007a, b). Using the Biolog-GN microplates to analyze the seasonal evolution of microbial communities in four lakes, Grover & Chrzanowski (2000) also observed strong relative responses of carbohydrates in the warm season, in periods of severe phosphorus deficiency. Our nP media logically corresponded to higher utilization of nitrogen-free carboxylic acids, such as galacturonic acid and pyruvic acid. Carboxylic acids are frequent in aquatic ecosystems. These organic compounds, products of bacterial fatty acid catabolism (Christian & Lind, 2007) and of photochemical transformation of dissolved organic matter (Bertilsson & Tranvik, 2000), are efficiently mineralized or incorporated by bacteria. Algal exudation of organic compounds readily consumable by prokaryotic heterotrophs is probably greater in situations with high imbalance between nutrients such as nitrogen and phosphorus (Nagao & Miyazaki, 2002), while there are some indications that DOC produced in more productive systems might be of higher molecular weight and less susceptible to microbial attack (Cotner & Biddanda, 2002). This could explain the preferential use of polymers, such as Tween 80, in the NP microcosms. Finally, the appearance of aromatic compounds such as phenols as preferential substrates in the more depleted np microcosms supports our hypothesis of more specialized bacterial communities under such highly depleted nutritive conditions. Thus, in spite of the restricted number of substrates, the Biolog study indicated that the functional diversity of the communities in our microcosms clearly depended on the nutritive conditions.

The limits of carbon substrate utilization patterns for characterizing bacterial communities instead of culturable isolates have been underlined regularly (Konopka *et al*., 1998; Preston-Mafham *et al*., 2002). In particular, it is a culture-based method and the slow-growing species might not be included in the analysis. Moreover, the metabolic redundancy of species implies that changes in the response may only crudely represent the actual microbial population dynamics. Therefore, according to Konopka *et al*. (1998), it is unclear how this approach can be used to provide fundamental information on questions of microbial diversity. Conversely, Preston-Mafham *et al*. (2002) underlined that the EcoPlates use more ecologically relevant structurally diverse compounds and are likely to pick up those organisms usually missed through being swamped by faster growing r-selected species on GN plates. They suggested that greater relevance and analytical power might be achieved in microbial diversity analysis with the Ecoplates than with their alternatives. The very consistent patterns observed in our experiments confirm the usefulness of this technique for analyzing the functional response of microbial communities, even when considering the foregoing caveats.

Coupling between the genetic and the functional diversity of bacteria

Several studies used different approaches simultaneously to analyze bacterial diversity. The data obtained this way provided convergent (de Lipthay *et al*., 2004), complementary (Maila *et al*., 2005) or contrasted (Sala *et al*., 2005) results. In the present work, the genetic and the functional diversities of the bacterial communities were compared for the size fraction $< 3\,\mu m$. The CoA indicated the existence of a very significant correlation between the ordinations of the two types of diversity. The common structure of the two data sets clearly segregated the NP, nP and Np communities. It was not as easy to classify the np communities because one of the replicates was an outlier in every analysis. Nevertheless, these communities seemed to be between NP and Np communities. This uncertainty and the high variability in the np microcosms may be linked to the confrontation of two opposing factors: the medium richness and the N : P ratio. The small dissimilarity between the results of the two diversity analyses relies on the fact that Np and np communities were genetically close while in terms of potential functional diversity np communities were closer to NP communities than to Np communities. The few OTUs that are different between the Np and np communities may be responsible for the major differences in potential functional diversity that were observed. Two communities close in terms of species composition may have different potential functional diversities when the nutritive conditions are not the same. Yet, the clear segregation between the NP, nP and Np communities is here, again, in total agreement with the changes in interactions between algae and bacteria described by Danger *et al*. (2007b) in these three treatments. Thus, there are strong codependencies and clear feedbacks between stoichiometric constraints and nutrient load, genetic and functional diversity of microbial communities and the nature of interactions between primary producers and decomposers. Our results are in general agreement with those of Naeem *et al*. (2000), who simultaneously manipulated producer and decomposer diversity in freshwater microcosms. They observed that algal production was a joint function of both algal and bacterial diversity, while bacterial production was mainly dependent on bacterial

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diversity. Moreover, these relationships were not straightforward, as revealed by significant interactions between algal and bacterial diversity. Measures of organic carbon use by bacteria (Biolog plates) in these microcosms indicated that carbon use was the mechanism responsible for these results. The authors suggested the importance of carbon-nutrient coupling through stoichiometric relationships in these complex interactions of codependency between producers and decomposers.

Biodiversity and multifunctionality in the macrobial and microbial worlds

Natural bacterial communities might contain hundreds or thousands of species within a few milliliters of water (Curtis *et al.*, 2006). With a few exceptions, most laboratory studies used communities with < 20 species. Bell *et al.* (2005) measured how biodiversity affects the functioning of communities containing up to 72 species. As underlined by the authors, the asymptotic level of the bacterial respiration was not yet attained when using the 72 taxa, and further increases in diversity would continue to increase the level of ecosystem functioning. This suggests that a very large number of species are necessary to simulate the functioning of natural bacterial assemblage correctly. In our experiment, we observed a total of 87 OTUs. An estimation of the relationship between the number of DGGE analyses and the number of discrete bands suggests an asymptotic value of roughly 110 OTUs. Taking into account the low sensitivity of DGGE for revealing rare units that generally constitute the largest fraction of phylotypes within communities, we can assert that the number of phylotypes in our cultures was much higher than the detected number of distinct OTUs. Thus, cultivating a natural microbial sample with an algal strain probably constituted a convenient approach for studying the relationships between genetic and functional diversity within communities.

This might be particularly important for studying ecosystem multifunctionality. Hooper *et al.* (2002) underlined that taxonomic diversity and functional diversity are correlated in most experiments. Hector & Bagchi (2007) also demonstrated the existence of a positive relationship between the number of ecosystem processes and the number of species influencing the overall functioning. One reason is that the coexistence of species is partly dependent on niche differentiation and dissimilarity between life-history traits. Thus, increasing the number of coexisting species should lead to increasing functional diversity. However, one might argue that microbial communities, with their very high abundance and number of taxonomical units, high dispersal capacity and cosmopolitan distribution, high plasticity and lateral gene transfer between bacterial cells, do not necessarily mirror the functioning of the macrobial world (for a review, see O'Malley & Dupré, 2007). Our study highlights the importance of environmental factors in structuring both genetic and functional diversity. Our results demonstrate the strong predictability of community structure in response to environmental constraints and the coupling between genetic and functional diversity of the bacterial assemblages. These results are in good accordance with those of Fuhrman *et al.* (2006), who observed that annually reoccurring bacterial communities are highly predictable from ocean conditions. This strongly suggests that a significant and ecologically relevant fraction of bacterial communities is characterized by a lower level of redundancy than frequently thought, as documented for many macrobial communities. Moreover, this also means that species identity strongly matters in biodiversity and ecosystem functioning studies. Thus, a wide range of ecological conditions and ecosystem processes should be explored to understand the real significance of ecological redundancy or to analyze the importance of biological insurance hypothesis. This also supports Hector & Bagchi's (2007) results on the importance of biodiversity for maintaining ecosystem multifunctionality. Two broad perspectives emerge at this point. First, as underlined in recent studies (Fuhrman *et al.*, 2006; Prosser *et al.*, 2007), the application and testing of theories established previously for plants and animals to microbial communities is currently limited and will certainly represent a major challenge in the future developments of microbial ecology and fundamental ecology. Second, in the vein of the study of Hector & Bagchi (2007), it seems necessary to explore the role of biodiversity in ecosystem multifunctionality, as this has been carried out for particular ecosystem processes in the first generation of experiments on biodiversity and ecosystem functioning.

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Authors'contribution

J.L. and M.D. contributed equally to this work.

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