



## FEATURE ARTICLE

# Importance of rare and abundant populations for the structure and functional potential of freshwater bacterial communities

Katalin É. Szabó<sup>1,2</sup>, Paul O. B. Itor<sup>1</sup>, Stefan Bertilsson<sup>1</sup>, Lars Tranvik<sup>1</sup>,  
Alexander Eiler<sup>1,\*</sup>

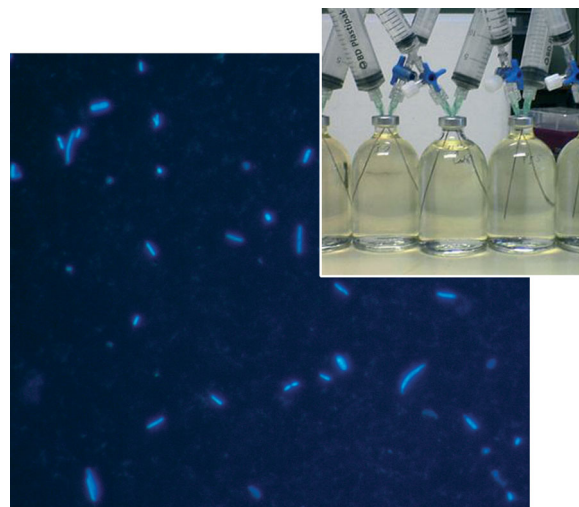
<sup>1</sup>Department of Ecology and Evolution, Limnology, Uppsala University, Husargatan 3 Box 573, Uppsala 75237, Sweden

<sup>2</sup>Hungarian Danube Research Station of the Hungarian Academy of Sciences, Jávorka Sándor utca 14, Göd 2131, Hungary

**ABSTRACT:** Lakewater microcosms were inoculated with freshwater bacterioplankton, to determine how the elimination of less abundant populations affects the structure and basic functional features (growth) of microbial communities. The number of bacteria added to individual microcosms varied from  $<1$  to  $2.6 \times 10^7$  cells. Cultures amended with  $11 \text{ mg C l}^{-1}$  of either isolated humic substances or phenol, as well as unamended controls, were studied in parallel. All cultures inoculated with 260 cells or more showed vigorous growth, whereas an inoculum size of 2.6 to 26 cells resulted in growth in the control and humic enrichment cultures only. All cultures were harvested at steady state within 14 d of inoculation. The biomass yield was only slightly affected by the dilution factor. The catechol 2,3-dioxygenase gene (encoding the enzyme responsible for starting the meta pathway of aromatic compound degradation) was detected in all phenol and in the least diluted humic enrichment cultures. Dominant members of the emerging bacterial communities were detected by terminal restriction fragment length polymorphism (T-RFLP) of PCR-amplified 16S rRNA genes. The number of detected community members was much higher in the humic treatment than in the phenol and control treatments. Based on the T-RFLP data, dilution of the inoculum significantly affected the resulting community composition ( $p < 0.0001$ ). Rare, opportunistic populations were apparently able to exploit the humic enrichment cultures. Phenol appeared to be detrimental to the most abundant members of the original inoculum, but promoted the growth of relatively rare species carrying the catechol 2,3-dioxygenase gene. Thus, community functioning following an environmental perturbation can depend on the presence of rare as well as abundant species.

**KEY WORDS:** Bacterioplankton · Dilution · 16S rRNA · T-RFLP · Catechol 2,3-dioxygenase · Bacterial growth potential

Resale or republication not permitted without written consent of the publisher



Dilution-to-extinction cultures of freshwater bacterial communities were incubated in unamended, phenol-enriched and humic substance-enriched lake water, to study the importance of rare and abundant bacteria for community performance. Biomass yield was not strongly affected by dilution, but exploitation of humic substances and resistance to phenol were restricted to less abundant populations, demonstrating that the performance of microbial communities may depend on rare as well as on abundant species.

Photos: Paul O. B. Itor (inset) & Katalin É. Szabó

## INTRODUCTION

Relationships between species diversity and ecosystem stability and function have been central topics in ecology for several decades (MacArthur 1955, May 1974, McNaughton 1977, Jones & Lawton 1995). The

widely discussed redundancy hypothesis tests if these relationships will depend on the degree of functional redundancy vs. complementarity among coexisting species (Lawton & Brown 1993). Functional redundancy is defined as the capability of several co-occurring bacterial taxa to perform the same function.

Recently, the effects of changing diversity on the functional potential of microbial communities has evoked intense interest, notwithstanding technical difficulties inflicted by the tremendous diversity of bacterial communities and unculturability of most species (Salonius 1981, Degens 1998, Garland & Lehman 1999, Franklin & Mills 2006). The dilution-to-extinction approach has frequently been used to manipulate microbial diversity for subsequent experimental studies because dilution will eliminate rare organisms from an initially diverse community. After regrowth with different dilutions of an original community as inocula, community composition will deviate from the original community in both species richness and composition, depending on the loss of populations during dilution (Franklin et al. 2001). The relationship between microbial diversity, functional potential and loss of biodiversity in the context of perturbation has been addressed in several studies that used serial dilutions of soil or sewage microbial communities as models (Salonius 1981, Degens 1998, Griffiths et al. 2001, Giller et al. 2004, Franklin & Mills 2006). These studies showed high functional redundancy in both ecosystems and found no consistent connection between biodiversity and ecosystem function or resilience. Both the tremendous richness of bacterial communities and the physiological versatility of bacterial populations were suggested to cause the lack of correlation between microbial community structure and function (Franklin & Mills 2006). For example, experimental studies reported considerable functional redundancy in natural bacterial communities (Langenheder et al. 2005, 2006), showing that differently composed communities perform similarly regarding, for example, degradation and mineralization of organic matter.

Most studies describing the effects of microbial diversity changes on community structure and functional potential used either soil or sewage communities as model systems (Degens et al. 2001, Franklin & Mills 2006). The aim of the present study was to assess the importance of rare and abundant populations in shaping community structure and community-level functions (the ability to utilize different dissolved organic substrates for growth) in microbial populations of native lakewater bacterioplankton. Three parallel treatments were incubated using sterile lake water as medium, and lake water amended with either humic substances or phenol. Both additions simulate a

perturbation event. Humic substances are complex molecules, heterogeneous in both their origin and molecular structure. Experimental studies have clearly demonstrated that at least part of this organic material is bioavailable (reviewed in Tranvik 1998). It has been argued that in future scenarios of global warming, loading of humic substances from the drainage area of lakes will increase (Tranvik & Jansson 2002). However, there is currently little information about the possible role of bacterial diversity in controlling the susceptibility of humic substances to microbial degradation and utilization in lakes.

Phenol enters the environment from anthropogenic sources (Van Schie & Young 1998) but it is also formed during the natural decomposition of organic polymers that contain aromatic structures. Bacteria play a key role in the degradation of phenol in soil, sediment and water, but the number of bacteria capable of utilizing phenol is usually a minor fraction of the total community (Hickman & Novak 1989). The majority of aromatic hydrocarbon compounds are converted by bacteria into catechol and protocatechuate, that subsequently undergo oxidative cleavage reactions via the ortho- or meta cleavage pathways (Cafaro et al. 2004). The first reaction of the meta pathway is started by catechol 2,3-dioxygenases (coded by the *xyIE* genes). Although microbial bioremediation is increasingly implemented, information on the diversity and functionality of the remediating communities is scarce (Whiteley & Bailey 2000), and little is known about the capacity of natural aquatic microbial communities to degrade aromatic compounds.

By amending the culture media with phenol and humic substances, the incubated communities were exposed to 2 different DOC sources: a mixture of complex molecules that can be highly bioavailable but is also recalcitrant, and phenol that is biodegradable by certain microbes but toxic to others. Here, we examined the effects of species loss on the growth of the microbial community and their combined ability to utilize these compounds with a specific focus on the respective roles of abundant and rare populations under the influence of perturbation.

## MATERIALS AND METHODS

**Experimental design.** Three series of dilution-to-extinction cultures were prepared with sterile lakewater from Lake Ekoln (a sub-basin of Lake Mälaren) as a growth medium. Ekoln is a eutrophic, dimictic lake with an average depth of 17 m and a total surface area of 20 km<sup>2</sup> (Eiler & Bertilsson 2004). A 20 l surface water sample was collected from the upper

0.5 m of the lake and transported to the laboratory within 30 min of sampling. Subsequently, the water was sequentially filtered through precombusted Whatman GF/C glass fiber filters and ultrapure Q-grade water-rinsed 0.2  $\mu\text{m}$  membrane filters (Pall Corp). Finally, the filter sterilized water was autoclaved twice (121°C for 20 min) with a 24 h interval. The sterile water was either used directly, or aseptically amended with 11.1 mg C l<sup>-1</sup> phenol or 12.2 mg C l<sup>-1</sup> humic substances. Hence, the original DOC content of the water (11.8 mg C l<sup>-1</sup>) was approximately doubled for these 2 treatments. The humic substances used were purchased freeze-dried from the Nordic Humic Society, reconstituted in ultrapure Q-grade water and then filtered 3 times through 0.2  $\mu\text{m}$  Supor membrane filters (Pall Corp). The pH of the amended and unamended sterile lakewater was balanced to the original 8.8 using 0.1 M sterile NaOH and 1 M sterile HCl.

Acid-rinsed and autoclaved glass bottles (total volume 110 ml) were used for the cultures. Each bottle was sealed with rubber stoppers, and 2 sterile syringes (20 and 10 ml) with needles were connected to each stopper with the needles projecting into the culture bottles and in contact with the culture liquid. This set-up allowed communication between the syringes and the culture liquid, thus enabling rapid sampling and a total cubic capacity of 140 ml. Aliquots (117 ml) of the different types of media were aseptically added to these culture bottles. Part of the medium was thus contained in the syringes, which facilitated sampling without introducing a gradually increasing headspace in the bottles.

A bacterial inoculum was collected from the same sampling site in Lake Ekoln using a sterile 20 l bottle. The sample was transported to the laboratory within 30 min of sampling and immediately filtered through precombusted Whatman GF/C filters to eliminate larger organisms, including bacterivores. Three 9-step dilution series were prepared for the respective sterile culture media. Three replicate cultures of each dilution (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-10</sup>) were prepared for each treatment (control, phenol, humic). Inoculum aliquots of 13 ml were added to the culture using the sterile 20 ml syringes attached to each bottle. The inoculum was mixed with the sterile media by repeated exchange of liquid between syringes and bottle, followed by vigorous shaking. All bottles were incubated in darkness at 23°C (ambient water temperature for the sampling date). Microscopic counts of the bacterial abundance in the original inoculum suggested that the 10<sup>-8</sup> dilution should contain an average of 2.6 cells and, consequently, we did not expect growth in the lowest dilution (10<sup>-10</sup>).

**Analysis of bacterial growth and biovolume.** Sub-samples (2 ml) were collected from the bulk medium in the bottles at 1 to 2 d intervals with the aid of 2 syringes, after mixing the cultures by shaking the culture bottle and by repeated exchange of liquid between both syringes and the bottle. Since 1 syringe contained excessive inoculated media the culture volume in the bottles could be kept constant. The samples were preserved by adding borax buffered formaldehyde to a final concentration of 2%. Bacterial abundance was monitored by flow cytometry of Syto 13 stained cells (del Giorgio et al. 1996), following a slightly modified protocol (Fogel et al. 1999). The maximum abundance value measured using blank controls was 4000 cells ml<sup>-1</sup>, hence values below this were regarded as background noise. Bacterial biovolumes were analyzed by fluorescence microscopy and image analysis of cells stained with DAPI (Porter & Feig 1980). Digital images acquired with a IDX1200 CCD camera (Nikon) were transferred to the Easy Image Analyzer 2000 software for volume estimates using the equation  $V = 4\pi(w/2)^3/3 + (l - w)\pi(w/2)^2$ , where  $l$  = length and  $w$  = width. Biovolumes were converted to biomass according to Loferer-Kröbächer et al. (1998). For details, see Eiler et al. (2003).

**DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis.** Cells from early stationary phase cultures were harvested by filtering approximately 100 ml of the culture onto 0.2  $\mu\text{m}$  membrane filters. Harvesting of the cultures was carried out after various incubation times since cultures with less dilute inocula reached stationary phase earlier than the more diluted cultures. Cultures were considered to be in stationary phase when 2 consecutive steady abundance values were measured. Filters were stored at -80°C until analysis and 20 ml of the filtrate was stored frozen for subsequent DOC and phenol analysis. DNA extraction was carried out using Ultraclean Soil DNA extraction kit (MoBio Laboratories). Frozen filters were cut into pieces, directly added to the bead tubes of the kit, and the procedure continued according to the manufacturer's instructions for maximum yield. Extracted nucleic acids were quantified and sized by agarose gel electrophoresis, ethidium-bromide staining and UV transillumination (Eiler & Bertilsson 2004). DNA concentrations varied between 0.2 and 10 ng  $\mu\text{l}^{-1}$ . DNA extracts were used as templates for PCR amplification of 16S rDNA with the bacterial primers 27-forward (Vergin et al. 1998) labeled with hexachlorofluorescein (HEX) and 519-reverse labeled with 6-carboxy-fluorescein (FAM) (Lane et al. 1985). Each reaction contained between 1 and 10 ng DNA template, 200  $\mu\text{m}$  of each deoxynucleoside tri-

phosphate, 100 nM of each primer, 0.25 U Taq polymerase and reaction buffer (DyNAzyme II Finnzymes OY). Thermocycling was carried out with a Stratagene Robocycler using an initial 3 min denaturation at 94°C, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min followed by a final 7 min extension step at 72°C. Between 2 and 8 replicate reactions were carried out for each sample. Pooled PCR products were digested with mung bean nuclease to eliminate single stranded PCR products (Egert & Friedrich 2003), followed by a purification and concentration using Qiaquick PCR purification kit (Qiagen). The PCR-product concentration in the eluate was analyzed by agarose gel electrophoresis and comparison to a Low DNA Mass Ladder (Invitrogen). Approximately 50 ng PCR product from each sample were digested with the restriction endonuclease *Hha*I (Invitrogen), using 10 µl reaction volumes. Reactions with PCR product, enzyme and buffer were incubated at 37°C for 16 h according to the manufacturer's instructions. Terminal fragments were sized by electrophoretic separation and detection on an ABI 3700 96-capillary sequencer after addition of a 500 bp ROX-labeled size marker (Applied Biosystems) to each sample. In some cases, samples were excluded from further analyses, because PCR amplification did not result in any product. The size and quantity of terminal restriction fragments were analyzed using GeneScanView 1.2/4 software (CRIBI, University of Padova) as previously described (Eiler & Bertilsson 2004). The lower cutoff for T-RFs to be included in the comparative analysis was 1 and 2.5% of the total peak area for the HEX- and FAM-labeled fragments, respectively. Binary data of presence or absence of individual T-RFs was used to calculate a similarity matrix ( $D_{xy}$ ) from 53 incubations according to the Sorensen equation:  $D_{xy} = 2N_{xy}/(N_x + N_y)$ , where  $N_x$  and  $N_y$  represent the number of OTUs either in incubation  $x$  or  $y$ , respectively, and  $N_{xy}$  is the number of OTUs present in both incubations. Semistrong hybrid nonmetric multidimensional scaling (NMDS) was performed by using Statistica (Statsoft) to reduce the multidimensional similarity matrix to 2 dimensions (Eiler et al. 2003).

**Catechol-2,3-dioxygenase survey.** The presence or absence of the *xylE1* gene coding for catechol-2,3-dioxygenase was assessed by PCR according to Mesarch et al. (2000), with minor modifications. Each reaction was carried out in 20 µl volumes, using 1 ng DNA template, 2.5 mM MgCl<sub>2</sub>, 200 µM of each deoxyribonucleoside, 1 U Taq polymerase with buffer (Invitrogen) and 0.25 µM of the primers 5'-CGACCTGATC(AT)(CG)CATGACCGA-3' and 5'-T(CT)AGG TCA(GT)(AC)ACGGTCA-3' (Mesarch et al. 2000).

Products of the correct size were identified using the Gel-Pro Analyzer version 3.1 and comparison to a 100 bp ladder (Invitrogen) and a positive control from a catechol-2,3-dioxygenase-positive *Pseudomonas fluorescens* strain CCUG32444 (Wikström et al. 1996). The detection limit for this assay is between 10<sup>2</sup> and 10<sup>3</sup> gene copies (Mesarch et al. 2000). Each DNA extract was analysed twice to rule out stochastic amplification in the PCR as a confounding factor.

**Analysis of DOC and phenol content.** DOC was analysed both in the original filter-sterilized lake water media and in each sample at the harvest date. All samples for DOC analyses had been filtered through 0.2 µm Supor membranes to remove cells and other particulate organic matter. Aliquots (6 ml) were acidified with 100 µl of 1.2 M HCl, purged with CO<sub>2</sub>-free air for 6 min to remove inorganic carbon, and subsequently analysed for total organic carbon by high-temperature catalytic oxidation and infrared detection using a Shimadzu TOC-5000 instrument.

Phenol concentrations were measured by reverse-phase HPLC, using a Merck-Hitachi L-4000A liquid chromatography system equipped with a Zorbax Eclipse-C18 column. The system was operated under isocratic conditions at 1 ml min<sup>-1</sup> with 70 v/v% 10 mM K<sub>2</sub>HPO<sub>4</sub> and 30 v/v% methanol as the mobile phase. Analytes were detected by absorbance at 270 nm. Standard curves were prepared by spiking autoclaved and 0.2 µm filtered lake water with 0.5 µM to 1 mM phenol. The regression curve ( $y = 0.2209x - 0.0049$ ,  $R^2 > 0.99 = 1$  or similar) was based on 19 samples. Detection limit (5 µM (0.47 mg l<sup>-1</sup>) phenol) was estimated visually, as the smallest visually detectable peak based on at least 3 replicates of a range of samples containing a low concentration (0.5 to 50 µM) of phenol.

**Statistical analysis.** Single-factor analysis of variance (1-way-ANOVA, Statistica StatSoft) was used to assess treatment effects on early stationary phase ('harvest') cell abundance and to assess dilution-effects on phenol degradation capacity. Two-way analysis of variance (2-way-ANOVA, Statistica StatSoft) was used to detect significant effects of treatments and dilution on stationary phase biomass and on the number of terminal restriction fragments of the T-RFLP analyses. Effects were considered significant if  $p < 0.05$ . Tukey's honest significant difference (HSD) test was used to distinguish between significantly different treatment levels. A group (level) was defined as homogenous when  $\alpha < 0.05$ . Mantel's test was used to correlate a dissimilarity matrix of bacterial community composition with a dissimilarity matrix that simply separates dilution factors of the inoculum for each of the 3 treatments.



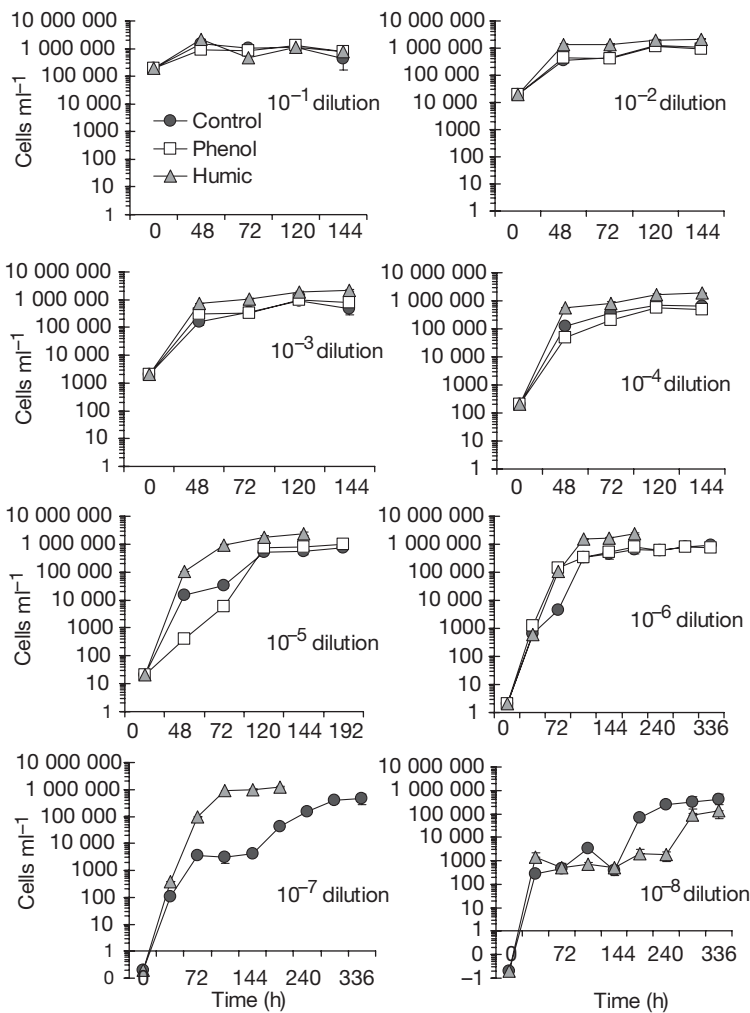


Fig. 1. Change in bacterial abundance as a function of time in cultures of different dilutions. Data points = median values, with maximum and minimum values of triplicate cultures. Note different timescales for the different dilutions

## RESULTS

### Bacterial growth dynamics

All cultures, irrespective of treatment, showed vigorous growth up to an inoculum dilution factor of  $10^{-6}$ . The stationary phase was reached within 2 to 8 d of inoculation (Fig. 1). As expected, cultures with less dilute inocula reached the stationary phase earlier than the more dilute cultures. Bacterial growth at the higher dilutions ( $10^{-7}$  and  $10^{-8}$ ) was only observed in the humic-amended treatments and the controls. No growth was observed in any of the  $10^{-10}$  dilutions. Humic substance amended cultures reached the stationary phase 1 or 2 d earlier than the corresponding dilutions in the other 2 treatments.

### Biomass yield

Stationary phase bacterial cell abundance was highest in the humic treatments, with a maximum abundance of  $2.8 \times 10^6$  cells  $\text{ml}^{-1}$ . Cell abundances were on average 3 times lower in the 2 other treatments with a range of  $0.32$  to  $1.44 \times 10^6$  cells  $\text{ml}^{-1}$  for the phenol treatment and  $0.49$  to  $1.24 \times 10^6$  cells  $\text{ml}^{-1}$  for the control treatment. The stationary phase cell abundance values were similar between the different dilutions within the phenol and control treatment (control:  $F = 2.21$ ,  $p = 0.104$ , 1-way ANOVA,  $df = 7$ , phenol:  $F = 1.22$ ,  $p = 0.359$ , ANOVA,  $df = 5$ ), whereas cell abundances in the humic amended cultures were clearly influenced by the dilution factor ( $F = 20.8$ ,  $p < 0.0001$ , ANOVA,  $df = 7$ ), resulting in very low abundance values at  $10^{-8}$  dilution. The culture-average cellular biovolumes were generally much lower in the humic treatment (range  $0.075$  to  $0.184 \mu\text{m}^3$ ) than the control (range  $0.032$  to  $0.265 \mu\text{m}^3$ ) and phenol-amended cultures (range  $0.134$  to  $0.314 \mu\text{m}^3$ ). Both the treatment type and the dilution factor had a significant effect ( $F = 11.76$ ,  $p < 0.001$ , 2-way ANOVA) on the bacterial biomass yield (Fig. 2), with the humic treatment cultures displaying significantly higher biomass yields ( $\alpha = 0.05$ ,  $df = 47$ , 2-way ANOVA with Tukey HSD test). Maximum biomass was observed at intermediate dilutions. For example, the maximum biomass was achieved in the  $10^{-5}$  dilutions in the control and phenol treatments, and in the  $10^{-6}$  dilution in the humic treatment (Fig. 2).

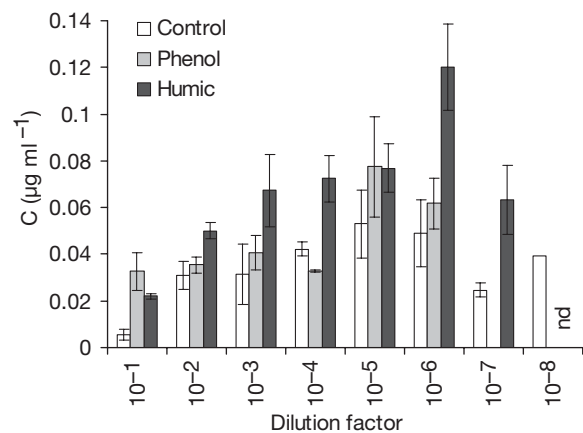


Fig. 2. Biomass of early stationary phase cultures in the 3 treatments. Columns indicate average values, bars standard deviations of triplicate cultures. nd = not detected: 2 humic cultures showed growth at  $10^{-8}$  dilution; however, the scarcity and tiny size of the cells made it impossible to make a reasonably accurate estimate of their biovolume and biomass

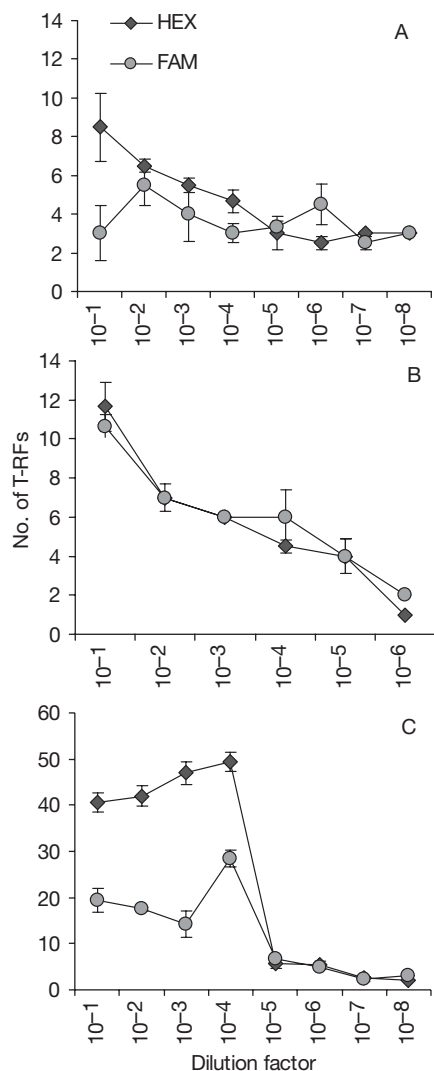


Fig. 3. Average ( $\pm$ SD;  $n = 3$  cultures) number of T-RFs in the 3 treatments: (A) control, (B) phenol and (C) humic. Scale of y-axis differs in (C)

### Bacterial community composition

The total number of detected T-RFs was 139 in the Hex-labeled and 82 in the Fam-labeled T-RFLPs. Both the dilution factor and the treatment type had a significant effect on the number of T-RFs in the Hex-labeled ( $p < 0.001$ , 2-way ANOVA with Tukey HSD analysis,  $\alpha = 0.05$ ,  $df = 38$ ) and the Fam-labeled ( $p <$

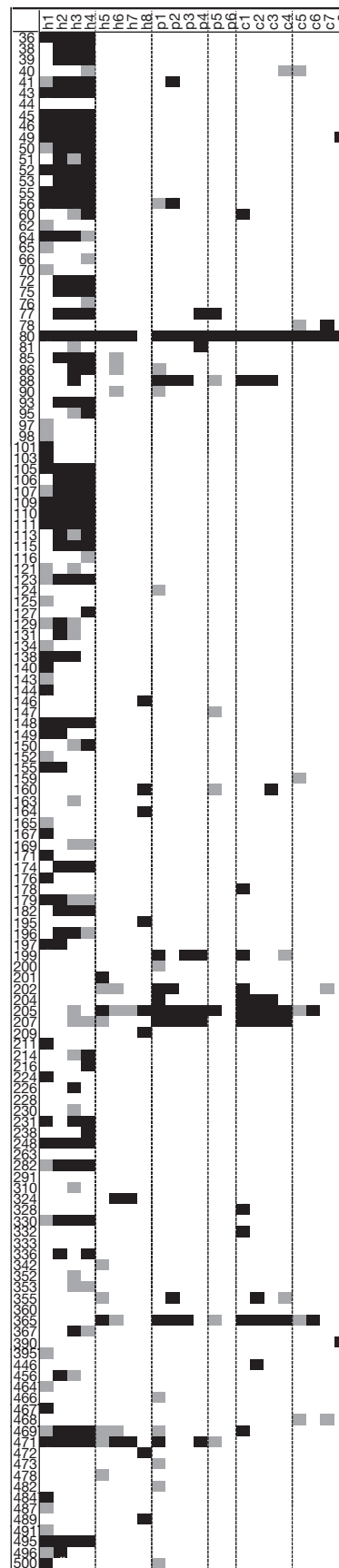


Fig. 4. Comparison of presence and absence of all T-RFs using HEX-labeled primer 27f in all treatments. Black shading = treatments where T-RF was detected in at least 50% of replicates; gray = T-RF detected in less than 50% of replicates in each treatment; h = humic, p = phenol, c = control treatments, treatment designations are followed by dilution factor of inoculum, where 1 =  $10^{-1}$ , 2 =  $10^{-2}$ , 3 =  $10^{-3}$ , etc. Dilutions resulting in no PCR-product are not shown

0.001, 2-way ANOVA with Tukey HSD analysis,  $\alpha = 0.05$ ,  $df = 37$ ) community analyses. The number of detected T-RFs in the control and phenol cultures showed a continuous decrease along the dilution gradient, with low dilutions resulting in higher T-RF numbers (Fig. 3A,B), although this decrease was less pronounced in the control cultures. In the humic treatment, there was an abrupt shift in the number of detected T-RFs between 2 of the intermediate dilutions (Fig. 3C). The low dilution treatments had a higher ratio of unique T-RFs than the high dilution treatments, and in the humic cultures, this ratio was as high as 80%. In the phenol series, only 2 dominant T-RFs were unique for the higher dilutions, while this was 20 and 25% in the humic and control cultures, respectively.

Analyzing the distribution of the T-RFs between the 3 different treatments, we found that 16 T-RFs occurred in all treatments, 15 were unique to the control, 13 to the phenol and 141 to the humic cultures; 5 T-RFs were shared between the control and phenol treatments, 4 between the control and humic treatments, and 15 between the humic and the phenol

treatments. Thus, the humic cultures did not only show the highest number of T-RFs, but also had the highest ratio of T-RFs that did not appear in the other 2 treatments. A single T-RF with a fragment length of 80 bp was observed in all treatments except in the highest dilution of the humic treatment (Fig. 4). A marked community change between the low and high dilutions of the humic cultures is also apparent in Fig. 4. Also in the other 2 treatments most T-RFs disappeared between the  $10^{-4}$  and  $10^{-5}$  dilution (Fig. 4).

The composition of emerging bacterial communities in the different cultures was analysed by NMDS (Fig. 5). In the humic substance-amended treatment, cultures prepared from both the least dilute inoculum ( $10^{-1}$ ) and the other less dilute inocula ( $10^{-2}$  to  $10^{-4}$ ) formed distinct groups, with high similarity values between the individual cultures (Fig. 5A). Although the higher dilutions did form one group, replicates of each dilution showed high dissimilarities from each other. Also in the phenol-amended cultures the high ( $10^{-4}$  to  $10^{-6}$ ) and low ( $10^{-1}$  to  $10^{-3}$ ) dilution treatments

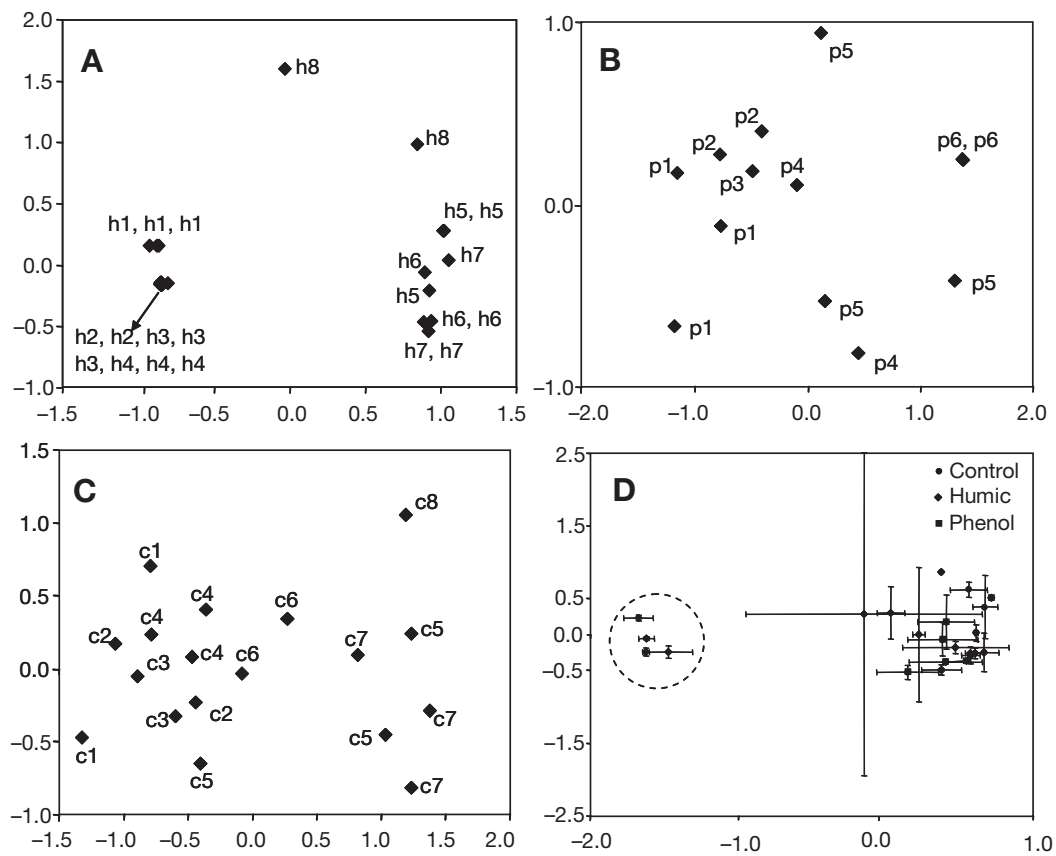


Fig. 5. NMDS based on combined restriction pattern of FAM- and HEX-labeled T-RFLPs. (A) Humic, (B) phenol, (C) control cultures; numbers indicate dilution factors, where  $1 = 10^{-1}$ ,  $2 = 10^{-2}$ ,  $3 = 10^{-3}$ , etc. (D) Combined T-RFLP pattern of all 3 treatments; values are means ( $\pm$ SD); dashed circle indicates humic treatments with low dilution factor

clustered separately (Fig. 5B), which was also supported by *K*-means cluster analysis (data not shown). The control treatment displayed some community clustering with dilution: the  $10^{-1}$  to  $10^{-4}$  dilution cultures were grouped together, the majority of the  $10^{-5}$  to  $10^{-8}$  dilutions were distant from the low dilutions (Fig. 5C). Using Mantel's test, a significant correlation between dilution factor and community dissimilarities could be observed in all treatments ( $p < 0.0001$ ;  $r = 0.64$  in the humic treatment;  $r = 0.50$  in the control treatment;  $r = 0.63$  in the phenol treatment). Comparing all samples, we found that the lower dilutions of the humic cultures were clearly separated from all other communities (Fig. 5D). The remaining treatments showed some grouping according to dilution, but less according to treatment type.

### Phenol degradation pattern of phenol-enrichment cultures

The gene for catechol 2,3-dioxygenase was detected in all phenol-enrichment cultures where growth was recorded ( $10^{-1}$  to  $10^{-6}$ ). The gene was also detected in the humic substance-amended cultures prepared from the least dilute inocula ( $10^{-1}$ ) and was not detected in any of the other cultures. There was no change in DOC content during the incubation for any of the treatments (data not shown). The phenol concentration in the phenol-amended cultures decreased down to 84 % of the original concentration. The concentration of phenol in the cultures with bacterial growth ranged from 9.29 to 11.02 mg C l<sup>-1</sup> at the time of harvesting. Phenol concentration stayed constant in sterile controls until the end of the experiment. There was no correlation between dilution factor and the percentual change in phenol concentration ( $p = 0.17$ ,  $F = 1.9$ , 1-way ANOVA,  $df = 5$ ).

## DISCUSSION

A range of studies, largely focused on plant communities in the terrestrial environment (e.g. Giller et al. 2004), suggests that increased species richness results in increased ecosystem function and stability (Loreau et al. 2001). Studies on bacteria support this pattern, in particular regarding stability (McGrady-Steed et al. 1997, Naeem & Li 1997, Langenheder et al. 2006), although several studies have reported the absence of consistent effects of soil microbial biodiversity on soil function (Degens 1998, Griffiths et al. 2000, 2001). In the present study, the elimination of less abundant species had different effects on the community composition as well as on some growth features of bacterial

communities, depending on the treatment. In both the control and phenol treatments, a gradual decrease in peak richness with increasing dilution could be observed, whereas a rapid decrease was observed in the humic treatment between dilutions of  $10^{-4}$  and  $10^{-5}$ . Based on the distinct community developing in the humic treatment with inocula ( $10^{-1}$  to  $10^{-4}$ ) where most rare populations were expected to remain, we propose that opportunistic but rare species ( $<10^6$  cells l<sup>-1</sup> in the original inoculum) were able to thrive in the humic-amended cultures. The number of T-RFs did not decrease up to the  $10^{-4}$  dilution. However, it is not known if this indicated a real stability of the richness in these samples or was rather an artefact of the fingerprinting method. T-RFLP has some well-known limitations, such as PCR bias (reviewed in Dorigo et al. 2005, Franklin et al. 2001 and references therein), the saturation of detected richness at approximately 35 T-RFs (Loisel et al. 2006) and the resulting underestimation of species richness. Nonetheless, T-RFLP has the advantages of high resolution and digital output, and provides a high level of insight into the structure of microbial communities (Marsh 1999).

The doubling of DOC concentration caused by the addition of humic substances supported the growth of a diverse subset of the original community, as shown by the large number of unique OTU-s in the humic cultures compared with the other 2 treatments. Humic substances do not conform to any defined structure, molecular weight or composition (Tranvik 1998), but it is reasonable to assume that the bioavailable fraction of humic substances is sufficiently heterogeneous to provide a variety of growth substrates which should be able to support the growth of a great diversity of heterotrophic populations. The rapid loss of T-RF number between  $10^{-4}$  and  $10^{-5}$  dilution indicates that the abundance of these populations was in the order of  $10^6$  cells l<sup>-1</sup> in the original sample. After these populations were diluted out of the inoculum, the communities changed dramatically (Fig. 5, NMDS). However, the biomass was at its maximum in the  $10^{-6}$  dilution indicating that the remaining populations were able to efficiently exploit the humic substances as growth substrates. This high biomass yield at this dilution could also have been caused by the decreased interspecific competition, as suggested by Franklin et al. (2001). Both the abundance and the biomass yield started to decrease at  $10^{-7}$  dilution, suggesting that the remaining (most abundant in the inoculum) populations could not perform as well as the more diverse communities.

Despite their gradual decrease in T-RF richness, the phenol treatment cultures showed a steeper decrease in T-RF numbers than the control treatment. The elimination of rare species had little effect on the general



ecosystem features such as biomass yield. Both the number of T-RFs and the total biomass were similar in both the phenol and the control treatments in the  $10^{-1}$  to  $10^{-6}$  dilutions. However, bacterial growth could only be detected up to the  $10^{-6}$  dilution in the phenol amended cultures, whereas in the other 2 treatments (controls and humic substance amended), growth could be detected in dilutions up to  $10^{-8}$ . These results indicate that phenol acted as a selective force, and that bacterial growth at phenol concentrations that are elevated in comparison with natural surface waters, probably depended on the presence of populations with a cell concentration of less than  $10^8$  cells  $l^{-1}$  in the original inoculum, corresponding to an addition of at least 260 cells. The capability of a narrow subset (as indicated by the low number of T-RFs) of the native freshwater bacterioplankton community to degrade phenol is also corroborated by the presence of the gene for catechol 2,3-dioxygenase and the decrease in added phenol concentration in all phenol treatments with bacterial growth.

These results suggest that some ecosystem functions, such as tolerance to aromatic pollutants (e.g. phenol) or the degradation of certain humic substances, may be affected by species loss at very low richness. Similarly, Salenius (1981) and Franklin & Mills (2006) found that ecosystem functions are affected when richness is decreased below a critical level, at which communities are just above extinction point. The source and type of DOC plays a determining role in shaping bacterial community composition (Riemann et al. 2000, Pinhassi et al. 2004), as was also supported by the preferential proliferation of very distinct taxa from a uniform starting inoculum in the phenol- and humic-amended cultures. The presence of a large pool of rare species capable of thriving on different fractions of humic substances, and a smaller pool of relatively rare species either capable of phenol degradation or tolerance, was demonstrated in this study. We conclude that ecosystem functioning would most probably be adversely affected by simultaneous loss of microbial richness and perturbation in the original lake ecosystem.

Functional differences among bacterial species, in particular niche complementarity, represent the foundation for relationships between species richness and ecosystem processes (Lawton & Brown 1993, Tilman 1999). It appears that the most abundant populations in our inoculum were capable of degrading a range of organic substrates and producing high biomass yields even at high dilutions. However, these dominant members of the inoculum did not grow in the phenol amended cultures, suggesting that more specific functions, such as the ability to withstand phenol-induced toxicity, were limited to the less

abundant populations. This illustrates that species that are redundant in a specific community at a given time under certain circumstances, may well have unique functions that may become crucial under different conditions. This agrees with the report of Griffiths et al. (2000) that coupling between structure and function in microbial communities may depend on the type of function and the initial community. The performance of microbial communities following an environmental perturbation, illustrated in this study by the transfer of the inoculum communities to a new medium, may depend on the presence of rare as well as abundant species.

*Acknowledgements.* We thank J. Johansson for technical support, S. Langenheder for providing the humic substances and helpful communications, E. Lindström for helpful discussions, S. Allen for support in the laboratory and M. Taylor for English revision. This work was supported by grants from the Swedish Research Council to L.T. and S.B., and the Hungarian State's Eötvös Postdoctoral Scholarship of the Hungarian Scholarship Board to K.É.S.

#### LITERATURE CITED

- Cafaro V, Izzo V, Scognamiglio R, Notomista E, Capasso P, Casbarra A, Pucci P, Di Donato A (2004) Phenol hydroxylase and toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. *Appl Environ Microbiol* 70(4):2211–2219
- Degens BP (1998) Decreases in microbial functional diversity do not result in corresponding changes in decomposition under different moisture conditions. *Soil Biol Biochem* 30:1989–2000
- Degens BP, Schipper LA, Sparling GP, Duncan LC (2001) Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? *Soil Biol Biochem* 33:1143–1153
- del Giorgio PA, Bird AF, Prairie YT, Planas D (1996) The flow cytometric determination of bacterial abundance in lake plankton with the green acid stain Syto 13. *Limnol Oceanogr* 41:783–789
- Dorigo U, Volatier L, Humbert JF (2005) Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Res* 39:2207–2218
- Egert M, Friedrich MW (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microbiol* 69(5):2555–2562
- Eiler A, Bertilsson S (2004) Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ Microbiol* 6(12):1228–1243
- Eiler A, Langenheder S, Bertilsson S, Tranvik LJ (2003) Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. *Appl Environ Microbiol* 69(7):3701–3709
- Fogel GB, Collins CR, Li J, Brunk CF (1999) Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population. *Microb Ecol* 38:93–113

- Franklin RB, Mills AL (2006) Structural and functional responses of a sewage microbial community to dilution-induced reductions in diversity. *Microbiol Ecol* 52: 280–288
- Franklin RB, Garland JL, Bolster CH, Mills AL (2001) Impact of dilution on microbial community structure and functional potential: comparison of numerical simulations and batch culture experiments. *Appl Environ Microbiol* 67:702–712
- Garland JL, Lehman RM (1999) Dilution/extinction of community phenotypic characters to estimate relative structural diversity in mixed communities. *FEMS Microbiol Ecol* 30:333–343
- Giller PS, Hillebrand H, Berninger UG, Gessner MO and 8 others (2004) Biodiversity effects on ecosystem functioning: emerging issues and their experimental test in aquatic environments. *Oikos* 104 :423–436
- Griffiths BS, Ritz K, Bardgett RD, Cook R and 8 others (2000) Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. *Oikos* 90:279–294
- Griffiths BS, Ritz K, Wheatley R, Kuan HL and 6 others (2001) An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. *Soil Biol Biochem* 33:1713–1722
- Hickman GT, Novak JT (1989) Relationship between subsurface biodegradation rates and microbial density. *Environ Sci Technol* 23(5):524–532
- Jones CG, Lawton JH (1995) Linking species and ecosystems. Chapman & Hall, New York
- Lane DL, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* 82:6955–6959
- Langenheder S, Lindström ES, Tranvik LJ (2005) Weak coupling between community composition and functioning of aquatic bacteria. *Limnol Oceanogr* 50(3): 957–967
- Langenheder S, Lindström ES, Tranvik LJ (2006) Structure and function of bacterial communities emerging from different sources under identical conditions. *Appl Environ Microbiol* 72(1):212–220
- Lawton JH, Brown VK (1993) Redundancy in ecosystems. In: Schulze ED, Mooney HA (eds) Biodiversity and ecosystem function. Springer-Verlag, Berlin, p 255–270
- Loferer-Krößbacher M, Klima J, Psenner R (1998) Determination of bacterial cell dry mass by transmission electron microscopy and densitometric image analysis. *Appl Environ Microbiol* 64:668–694
- Loisel P, Harmand J, Zemb O, Latrille E, Lobry C, Delgenès JP, Godon JJ (2006) Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. *Environ Microbiol* 8:720–731
- Loreau M, Naeem S, Inchausti P, Bengtsson J and 12 others (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294:804–808
- MacArthur R (1955) Fluctuations of animal populations and a measure of community stability. *Ecology* 36:533–536
- Marsh T (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* 2:323–327
- May RM (1974) Biological populations with nonoverlapping generations: stable points, stable cycles, and chaos. *Science* 186:645–647
- McGrady Steed J, Harris PM, Morin PJ (1997) Biodiversity regulates ecosystem predictability. *Nature* 390:162–165
- McNaughton SJ (1977) Diversity and stability of ecological communities: A comment on the role of empiricism in ecology. *Am Nat* 111:515–525
- Mesarch MB, Nakatsu CH, Nies L (2000) Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. *Appl Environ Microbiol* 66(2):678–683
- Naem S, Li S (1997) Biodiversity enhances ecosystem reliability. *Nature* 390:507–509
- Pinhassi J, Sala MM, Havskum H, Peters F, Guadayol OÒ, Malits A, Marrase C (2004) Changes in bacterioplankton composition under different phytoplankton regimes. *Appl Environ Microbiol* 70:6753–6766
- Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* 25:943–948
- Riemann L, Steward GF, Azam F (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* 66:578–587
- Salonius PO (1981) Metabolic capabilities of forest soil microbial populations with reduced species diversity. *Soil Biol Biochem* 13:1–10
- Tilman D (1999) The ecological consequences of changes in biodiversity: a search for general principles. *Ecology* 80(5): 1455–1474
- Tranvik LJ (1998) Degradation of dissolved organic matter in humic waters by bacteria. In: Hessen DO, Tranvik LJ (eds) Aquatic humic substances—ecology and biogeochemistry. Springer-Verlag, Berlin, p 259–278
- Tranvik LJ, Jansson M (2002) Climate change—terrestrial export of organic carbon. *Nature* 415:861–862
- Van Schie PM, Young LY (1998) Isolation and characterization of phenol-degrading denitrifying bacteria. *Appl Environ Microbiol* 64:2432–2438
- Vergin KL, Urbach E, Stein JL, DeLong EF, Lanoil BD, Giovannoni SJ (1998) Screening a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order *Planctomycetales*. *Appl Environ Microbiol* 64:3075–3078
- Whiteley AS, Bailey MJ (2000) Bacterial community structure and physiological state within an industrial bioremediation system. *Appl Environ Microbiol* 66(6): 2400–2407
- Wikström P, Wiklund A, Andersson AC, Forsman M (1996) DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. *J Biotechnol* 52:107–120

*Editorial responsibility: Josep Gasol, Barcelona, Spain*

*Submitted: July 14, 2006; Accepted: January 15, 2007  
Proofs received from author(s): March 23, 2007*