Environmental Microbiology (2006) 8(4), 625–638 doi:10.1111/j.1462-2920.2005.00939.x

# Replicability of dominant bacterial populations after long-term surfactant-enrichment in lab-scale activated sludge

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## Summary

**Bacterial communities were examined in replicate lab-scale activated sludge reactors after a period of several months of enrichment with non-ionic nonylphenol ethoxylate (NPE) surfactants. Four sequential batch reactors were fed with synthetic sewage, two of which received additionally NPE. Small subunit rDNA-derived denaturing gel gradient electrophoresis (DGGE) profiles and 16S rDNA clone libraries were dominated by clones of *Gammaproteobacteria* class. Sequences of the other codominant rDNA phylotypes observed only in DGGE from NPE-amended reactors were, respectively, associated with the Group III of the *Acidobacteria* phylum. Intriguingly, 16S rRNA content from abundant *Gammaproteobacteria* cells was unexpectedly low. In addition to *Acidobacteria*, rRNA-derived DGGE profiles were dominated by members of the order *Burkholderiales* (of the *Betaproteobacteria*) and of the genus *Sphingomonas* (a member of the *Alphaproteobacteria*). Specific oligonucleotide probes for the selected ribotypes were designed and applied for quantitative real time polymerase chain reaction and fluorescence *in situ* hybridization, confirming their dominance in treated reactors. The parallel abundance of unique phylotypes in replicate reactors implies a distinctive selection of dominant organisms, which are better adapted to specialized niches in the highly selective environment.**

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## Introduction

Two important questions in microbial ecology are whether functional stability of complex ecosystems entails a persistent microbial community and how do microbial communities react to variations in environmental conditions. These issues are especially relevant for engineered systems, where the diversity appears to be directly linked to process stability (Briones and Raskin, 2003; Curtis *et al.*, 2003)

Data from lab-scale experiments revealed highly dynamic bacterial communities performing in functionally stable anaerobic reactors (Godon *et al.*, 1997; Fernandez *et al.*, 1999; Zumstein *et al.*, 2000; McHugh *et al.*, 2004), aerobic activated sludge reactors (Kaewpipat and Grady, 2002) and compost reactors (Schloss *et al.*, 2003). Interestingly, methanogenic communities in anaerobic reactors (Fernandez *et al.*, 1999; Pender *et al.*, 2004), as well as communities of fluidized bed reactors treating aromatic compounds (Massol-Deyá *et al.*, 1997), communities from full-scale multistage process treating pharmaceutical wastewater (LaPara *et al.*, 2002) and nitrifying bacterial communities (Daims *et al.*, 2001; Rowan *et al.*, 2003) showed a more stable and reproducible behaviour.

Allowing the fact that the reactor size also appears to play a role in the community stability, an appealing account of the former observations, put forward by Curtis and Sloan, is that the better replication of the latter systems may be the result of the lower diversity of the more specialized communities (Curtis and Sloan, 2004).

With the purpose of addressing the issue of reproducibility in a systematic way, we have been studying the structure of bacterial communities in suspended growth systems subjected to selective feeding. In a previous report we have shown that the structures of bacterial communities in replicate lab-scale sequential batch reactors, which only differed in that one pair of replicates was fed with nonylphenol ethoxylate (NPE) in addition to synthetic sewage, were significantly reproducible at the level of broad group-specific small-subunit rRNA oligonucleotide probes (Lozada *et al.*, 2004). The system was selected because of the complex microbial degradation established for NPE and the high diversity of bacterial species that have the ability to grow using NPE as a

source of carbon and energy (Barberio *et al.*, 2001). Because of their extensive use as detergents, emulsifiers, dispersing and wetting agents, non-ionic surfactants such as NPE are widely released in the environment, mostly after secondary biological treatment. As a consequence of biological wastewater treatment, partial degradation occurs with the formation of recalcitrant intermediates, which are considered important environmental pollutants attributed to their toxicity and endocrine disrupting activity (John and White, 1998; Ferguson *et al.*, 2001). As the primary source of these compounds is considered to be the breakdown of NPE during sewage treatment, many efforts were made to understand the fate of NPE in biological treatment (Gross *et al.*, 2004).

With the aim of assessing the replicability of the bacterial community composition after a long period of selective feeding at higher levels of resolution, we report here a full quantitative account of dominant members of the stable communities in replicate NPE-amended reactors by using denaturing gel gradient electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA) of clone libraries, partial sequencing of SSU rDNA clones, fluorescence *in situ* hybridization (FISH) and quantitative real time polymerase chain reaction (qPCR).

We show that the dominance of few populations of distinct members of phylum *Acidobacteria* and of classes *Alpha*, *Beta* and *Gamma* of the *Proteobacteria* phylum were highly reproducible in replicate reactors subjected to selective regime by surfactant addition.

## Results

### *Amplified ribosomal DNA restriction analysis of the activated sludge microbial community enriched with NPE*

Four clone libraries were constructed with amplified nearly complete 16S rDNA sequences from semicontinuos activated sludge (SCAS) reactors fed with synthetic sewage with and without the addition of NPE. A total of 187 clones containing inserts of about 1450 bp were digested with restriction enzymes RsaI and HhaI. Clones having the same restriction fragment length polymorphisms (RFLP) patterns with both enzymes were grouped into an operational taxonomic unit (OTU), as they represent organisms with a median genetic distance of 95.6% (Moyer *et al.*, 1996). All clones analysed in control reactor libraries comprised OTUs that were observed just once or twice in each sample. In contrast, clones from NPE-treated reactors were characterized by a significant proportion of an OTU (S1A3 in SCAS1), with a frequency of 27% and two abundant OTUs (S3A1 and S3D1 in SCAS3), with frequencies of 18% and 10%.

### *Phylogeny of dominant* Gammaproteobacteria *phylotypes in SCAS*

Representative clones of the dominant ARDRA types in rDNA libraries from both NPE-amended reactors were selected for 16S rDNA sequence analysis. 16S rDNA from clone S1A3, representing the most abundant OTU in SCAS1, as well as S3A1 and S3D1, representing the two most abundant OTUs in SCAS3, exhibited a high degree of sequence similarity, and was related to uncultured environmental clones belonging to the class *Gammaproteobacteria* (Fig. 1). We have also sequenced the single clone from control reactor (S4A5) having the same ARDRA type of dominant clones in amended reactors, which resulted to be identical to S1A3, and a clone of similar, but not identical ARDRA type in reactor SCAS1, which was related to the *Betaproteobacteria* (Fig. 1).

All four closely related sequences of dominant *Gammaproteobacteria* clones from NPE-amended reactors became the focus group for specific probe design. Two probes, S-*-UGP-1230-a-A-18 and S-*-UGP-1291-a-A-18, were designed to target positions 1230–1247 and 1291–1308 in *Escherichia coli* numbering (Table 1). A BLAST search revealed that several closely related environmental clones also contained the probe sequence (Fig. 1).

In accordance with previous results from our laboratory, *Gammaproteobacteria* targeted by probe GAM42a represented less than 5% of total bacteria, whereas BET42a-targeted *Betaproteobacteria* accounted for *c.* 30% of all bacteria (Lozada *et al.*, 2004). The class-specific probes GAM42a/BET42a, which target, respectively, *Gammaproteobacteria* and *Betaproteobacteria*, vary by only one nucleotide at position 1033 of 23S rRNA (Manz *et al.*, 1992). In order to examine the possibility that the group of clones affiliated with the class *Gammaproteobacteria* yielded positive hybridization signal with probe BET42A (Lozada *et al.*, 2004) because of polymorphisms within probe-binding site in helix 42 of the 23S rRNA (Yeates *et al.*, 2003; Siyambalapitiya and Blackall, 2005), we have used the reverse complement version of probe S-*-UGP-1291-a-A-18 together with a universal 23S rRNA primer 23S-6F (Table 1) to amplify the fragment of the rRNA operon containing the target site of the probes. Partial sequences of the 16S rDNA portion and the 23S rDNA portion were generated from several clones. In all of these clones, the sequences 3′ from the primer S-*-UGP-1291-a-A-18 were found to be identical to the sequence of the clones S1A3, S3D1 and S4A5, and all had uracyl (U) at position 1033 of the 23S rRNA, i.e. complementary to adenine (A) of probe GAM42a.

### *Quantitative analysis of* Gammaproteobacteria

Unfortunately none of the newly designed taxon-specific probes for *Gammaproteobacteria* worked in FISH.

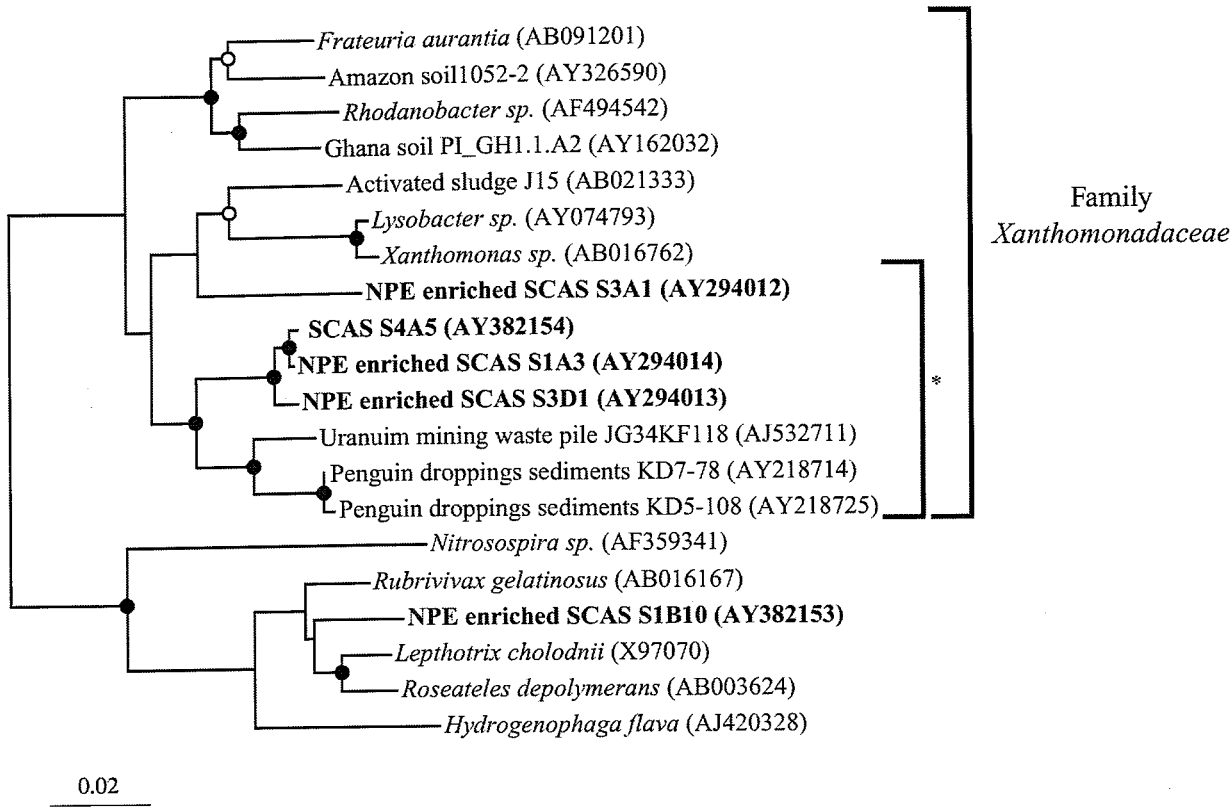


**Fig. 1.** 16S rDNA-based phylogenetic tree showing the relationship of dominant SCAS libraries clones to their closest relatives and other cultured and environmental members of *Beta* and *Gamma* classes of the *Proteobacteria*. The scale bar represents 2% estimated change per nucleotide. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbour joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested. The strains matching UGP probes designed in this study are indicated by an asterisk.

**Table 1.** Primers and probes used for PCR, DGGE, FISH and real-time PCR.

| Name[a] | Use | *Escherichia coli* rRNA gene positions | Reference or sequence (5′ to 3′), if designed for this study |
|---|---|---|---|
| S-D-Bact-0008-a-S-20 | PCR, cloning | 8–27 | Godon *et al.* (1997) |
| S-*-Univ-1492-a-A-18 | PCR | 1492–1509 | Godon *et al.* (1997) |
| 341F (GC clamp) | DGGE | 341–357 | Muyzer *et al.* (1993) |
| 534r | DGGE | 518–534 | Muyzer *et al.* (1993) |
| 984 (GC clamp) | DGGE | 968–984 | Heuer *et al.* (1999) |
| r1378 | DGGE | 1378–1401 | Heuer *et al.* (1999) |
| f930 | Real time PCR | 914–930 | Barberio *et al.* (2001) |
| f1114 | Real time PCR | 1099–1114 | Rainey *et al.* (1996) |
| S-*-Univ-1392-a-A-15 | Real time PCR, RT-PCR | 1392–1406 | Pace *et al.* (1986) |
| S-*-AcG3-1282-a-A-18 | Cloning | 1282–1299 | CTGAGACCGGTTTTTGCG |
| S-*-UGP-1291-a-A-18 | Real time PCR, cloning | 1291–1308 | ATCCGGACTGAGAGAGGT |
| S-*-UGP-1230-a-A-18 | Southern blot | 1230–1247 | ACCTCCCATTGTAGTACG |
| 31F | Cloning *Acidobacteria* | 15–31 | Barns *et al.* (1999) |
| S-*-OTU45-0720-a-A-20 | FISH | 720–739 | Juretschko *et al.* (2002) |
| 23S-6F | Sequencing γ-*Proteobacteria* | 559–578 | Antón *et al.* (1999) |
| 1930-r | Cloning γ-*Proteobacteria* | 1930–1947 | Ehrmann *et al.* (1994) |
| Bet948β | Cloning β-*Proteobacteria* | 931–948 | Gomes *et al.* (2001) |
| S-*-LARI-0470-a-A-18 | FISH | 470–487 | TACCGTCATCCTCCCGAG |
| Sph492[b] | FISH | 492–509 | Friedrich *et al.* (2002) |

**a.** Probes developed in this study were named according to the study by Alm and colleagues (1996)
**b.** Helpers were modified according to the retrieved sequence from DGGE. H433: ATCCCKGGTAAAAGAGC; H450 (modif): CCGGTACTGTCAT GTATC; H510 (modif): SSGCTGCTGGCACGRAGT; H528 (modif): CTAGCTCCCYYCGTATTACCG.

**Table 2.** Quantification of UGP in SCAS samples determined by quantitative real time PCR.[a]

| Reactor | Uncultured γ-*Proteobacteria*[b] | Total bacteria[c] | % of total bacteria |
|---|---|---|---|
| SCAS 1 | | | |
| rDNA | $4.6 \times 10^{11} \pm 0.5 \times 10^{11}$ | $3.3 \times 10^{12} \pm 0.5 \times 10^{12}$ | 14 ± 1 |
| rcDNA | $7 \times 10^{11} \pm 1 \times 10^{11}$ | $4.4 \times 10^{14} \pm 0.7 \times 10^{14}$ | 0.15 ± 0.02 |
| SCAS 2 | | | |
| rDNA | $1.8 \times 10^{11} \pm 0.2 \times 10^{11}$ | $3.2 \times 10^{12} \pm 0.9 \times 10^{12}$ | 6 ± 2 |
| rcDNA | $1.6 \times 10^{12} \pm 0.5 \times 10^{12}$ | $4.4 \times 10^{14} \pm 0.4 \times 10^{14}$ | 0.36 ± 0.09 |
| SCAS 3 | | | |
| rDNA | $1.7 \times 10^{11} \pm 0.3 \times 10^{11}$ | $1.1 \times 10^{12} \pm 0.3 \times 10^{12}$ | 16 ± 4 |
| rcDNA | $1.0 \times 10^{12} \pm 0.2 \times 10^{12}$ | $2.5 \times 10^{14} \pm 0.6 \times 10^{14}$ | 0.4 ± 0.2 |
| SCAS 4 | | | |
| rDNA | $6.8 \times 10^{10} \pm 0.9 \times 10^{10}$ | $3.6 \times 10^{12} \pm 0.4 \times 10^{12}$ | 2.0 ± 0.4 |
| rcDNA | $1.2 \times 10^{12} \pm 0.5 \times 10^{12}$ | $6 \times 10^{14} \pm 2 \times 10^{14}$ | 0.21 ± 0.05 |

**a.** The DNA concentration was measured as copies of 16S rDNA per litre ± standard deviation ($n = 3$).
**b.** Primers used were f930 and S-*-UGP-1291-a-A-18.
**c.** Total bacterial 16S rDNA and 16S rcDNA provide normalization for comparison between samples. Primers used were f1114 and S-*-Univ-1392-a-A-15.

Therefore, we developed a real-time quantitative PCR assay to measure the abundance of selected rDNA and rRNA across the four reactors relative to that of total bacterial rDNA and rRNA.

The PCR efficiencies were 0.80 and 0.87 for general bacterial and UGP primer systems. Table 2 depicts the copy number and percentages of bacterial rDNA and rRNA that were detected in the UGP-specific assay for samples in the four reactors. For comparison with 16S rDNA libraries, we have estimated percentage of 16S rDNA copy numbers without making any assumption regarding 16S rDNA copies per cell for total bacteria or for the targeted *Gammaproteobacteria*. The two NPE-amended reactors (SCAS1 and SCAS3) contained substantial amounts of UGP rDNA. Nested analysis of variance indicated that UGP population sizes were not significantly different among NPE-amended reactors ($P = 0.089$) but were significantly different from control reactors ($P < 0.001$). By contrast, the relative proportion of UGP at the rRNA level in NPE-amended reactors was substantially reduced to less than 1%, and not significantly different from control reactors.

### *Comparison of DNA- and RNA-derived DGGE profiles*

DNA- and RNA-derived DGGE profiles were performed in order to gain insight into the identities of predominant bacterial populations in the bioreactors. Comparison between DGGE patterns derived from rDNA and rRNA profiles showed marked differences in bacterial communities from all four reactors. In the case of rRNA only a small number of bands dominated DGGE profiles. As expected, both 16S rDNA and rRNA profiles exhibited large differences between NPE-amended reactors and control reactors, whereas replicate reactors showed less variability (Fig. 2).

Comparative DGGE analysis of 16S rRNA and 16S rDNA-derived profiles revealed that dominant DGGE band D was not detected in the rRNA-based PCR-DGGE profiles (Fig. 2). The identity of band D extracted from NPE-amended reactors was 100% similar to clone S1A3, and was confirmed by Southern blot of the DGGE using the specific S-*-UGP-1291-a-A-18 probe (data not shown). The low rRNA content observed in DGGE experiments

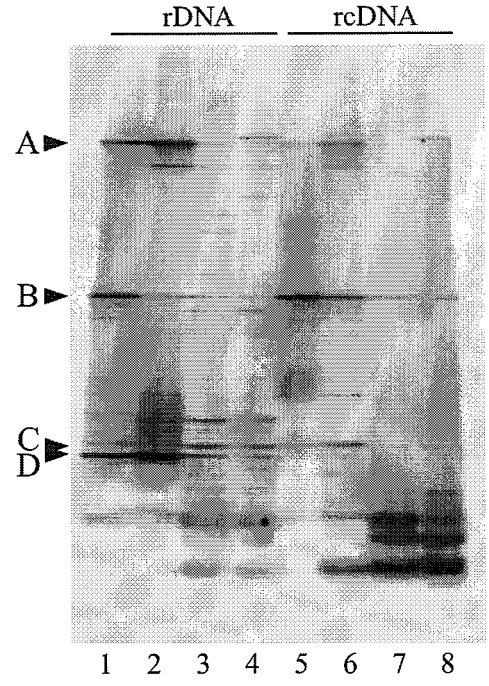


**Fig. 2.** Comparison between DGGE patterns of PCR and RT-PCR products of the V3 region from DNA (lanes 1–4) and rRNA (lanes 5–8) isolations of sludge from NPE-amended reactors (lanes 1, 2, 5 and 6) and control reactors (lanes 3, 4, 7 and 8). Bands A–D are discussed in the text.

was in agreement with the low abundance observed in previous FISH experiments, for fixed sludge targeted with probe GAM42a (Lozada *et al.*, 2004), and with the low ribosome number determined in quantitative PCR experiments, indicating that despite their abundance, *Gammaproteobacteria* cells exhibit a substantially low degree of metabolic activity.

### *Phylogenetic analysis and quantitation of other dominant members*

A 16S rDNA analysis was conducted to identify the bacterial species that dominated DNA- and RNA-derived profiles from NPE-amended reactors, identified as A–D in Fig. 2. All sequences of bands running at the same position, which were extracted from DGGE of both NPE-amended reactors were 100% identical, and were identified in terms of their closest relatives in GenBank database. Small subunit rDNA clone libraries were also screened by DGGE in order to relate dominant DGGE bands to specific clones in the libraries and obtain longer sequences for better phylogenetic placement.

The 16S rRNA sequences of DGGE band A from both NPE-amended reactors (corresponding to *E. coli* positions 341–534, including the V3 region) were closely related to members of the *Acidobacteria* phylum. To permit unambiguous phylogenetic identification of these phylotypes, we amplified the 16S rDNA fragment spanning positions 984–1401 (corresponding to *E. coli* numbering), which includes the V6–V8 region and run a DGGE (not shown). The sequence of one of the most dominant bands in these DGGE was also related to members of the *Acidobacteria* phylum, and was used to develop a specific probe (S-*-AcG3-1282-a-A-18) that targeted positions 1282–1299 (in *E. coli* numbering) of the small-subunit rDNA (Table 1). Other related environmental sequences were also targeted by the S-*-AcG3-1282-a-A-18 probe (Fig. 3A). Using our newly designed probe in conjunction with the *Acidobacteria*-specific primer 31f (Table 1), a 1250 bp 16S rDNA fragment was amplified from genomic DNA extracted from NPE-amended bioreactor sludge, cloned and sequenced. Linking of the clone identities to band A in DGGE gels (Fig. 2) confirmed that these sequences matched the dominant band A in both the rDNA- and rRNA-derived DGGE profile, and also matched the sequence from the DGGE band from the V6–V8 region, from which the probe sequence was retrieved. BLASTN program identified a number of similar 16S rDNA fragments (per cent similarity up to 92%), all of which have been identified as uncultivated bacteria belonging to the new, widely distributed Group III of *Acidobacteria* (Fig. 3A) (Quaiser *et al.*, 2003; Zhou *et al.*, 2003).

The presence of bacteria of the *Acidobacteria* phylum was further determined *in situ* by using cy3-labelled probe S-*-OTU45-0720-a-A-20 (Table 1, Fig. 3B–C). Consistent with the DGGE data, members of *Acidobacteria* were detected with a relative *in situ* abundance of 13% (Table 3).

The identity of dominant band B in rRNA-derived DGGE profile, revealed the presence of a phylogenetic lineage belonging to the order *Burkholderiales* (Fig. 4A). This result was confirmed by the use of a taxon-specific primer to amplify and clone a fragment of *c.* 940 bp, using a general bacterial specific primer S-D-Bact-0008-a-S-20 and the reverse complement of *Betaproteobacteria*-specific primer Bet948β (Table 1), which served as the reverse primer, after reverse transcription of rRNA from NPE-amended reactor sludge. The sequencing results confirmed that the amplicons were related to band B, as all sequenced fragments were 100% identical in the corresponding region.

Figure 4B–D shows that a large proportion *Betaproteobacteria*, detected with probe BET42a in our previous experiments (Lozada *et al.*, 2004) were also positive for our newly designed probe S-*-LARI-0470-a-A-18, which targets specifically members of the order *Burkholderiales* (Table 1). Cells that hybridized with probe S-*-LARI-0470-a-A-18 comprised 13.3% of all bacteria in NPE-amended sludge and only 3.2% of control reactors (Table 3).

Sequencing of DGGE band C resulted in assignment to a phylogenetic group belonging to *Alphaproteobacteria*. Clone S1D10 from NPE-amended sludge appeared to comigrate with band C in DGGE. A 758 bp 16S rDNA fragment of clone S1D10 was sequenced for phylogenetic analysis, showing 100% similarity with the DGGE band

**Table 3.** Relative abundance of dominant bacterial phylogenetic groups estimated from fluorescent *in situ* hybridization.

| | NPE-amended reactors[a] | | Control reactors | | | |
|---|---|---|---|---|---|---|
| Phylogenetic group | SCAS1 | SCAS3 | SCAS2 | SCAS4 | *n* | *P*[b] |
| *Acidobacterium* Group III | 12.0 ± 1.9 | 14.5 ± 5.5 | 0.3 ± 0.1 | 0.5 ± 0.3 | 10 | < 0.001** |
| *Betaproteobacteria* order *Burkholderiales* | 14.3 ± 7.0 | 12.5 ± 2.9 | 6.3 ± 11.1 | 0.0 ± 0.0 | 16 | 0.002** |
| *Alphaproteobacteria* gen. *Sphingomonas* | 10.0 ± 3.2 | 9.0 ± 5.7 | 0.2 ± 0.2 | 0.0 ± 0.0 | 16 | < 0.001** |

**a.** Mean percentage ± 95% confidence interval relative to DAPI-stained cells.
**b.** No significant differences between replicate reactors were found for any of the probes used.

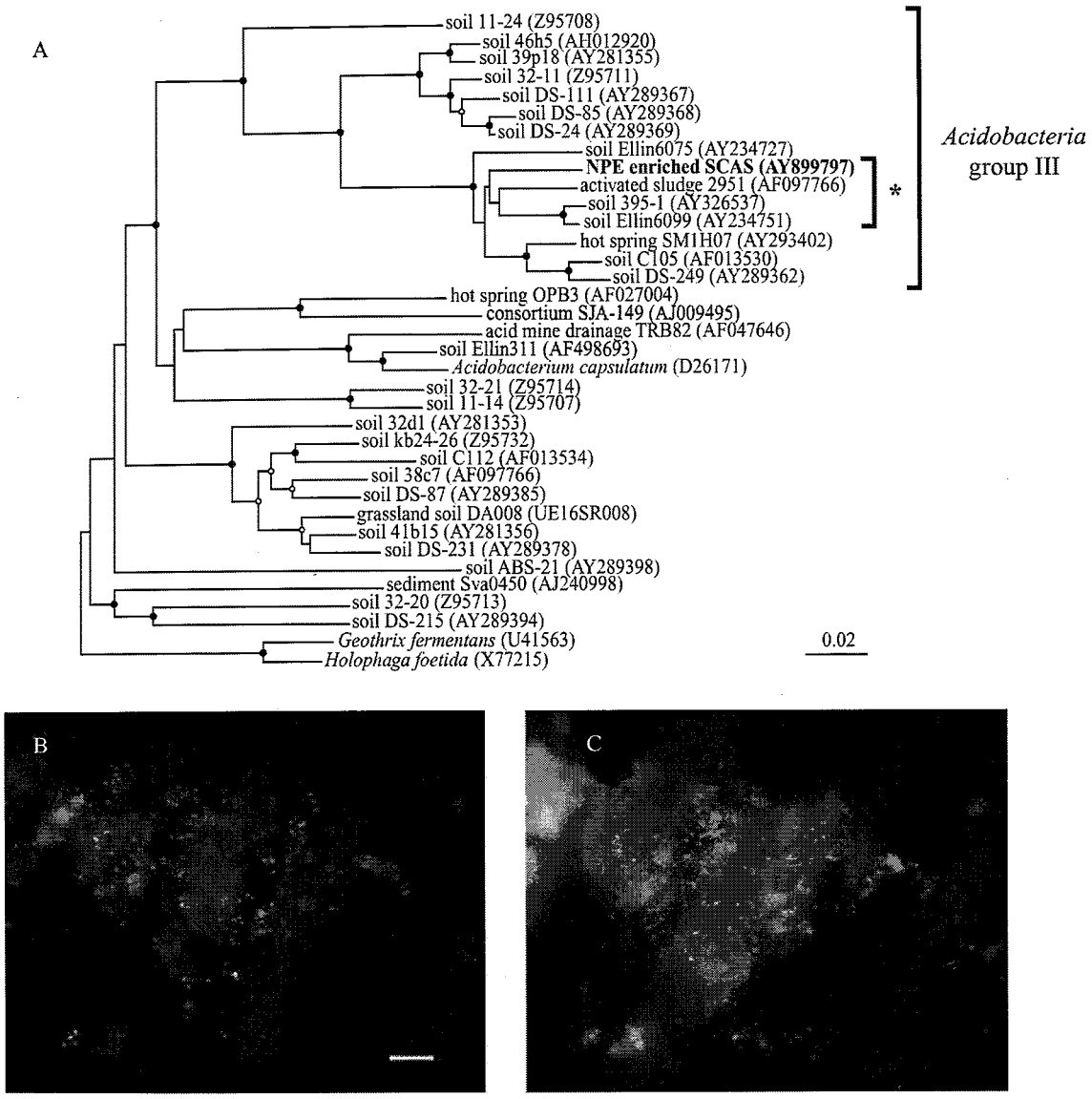


**Fig. 3.** A. Dendrogram showing the phylogenetic position of *Acidobacteria* clones obtained from amplification of genomic DNA from SCAS1, with primers 31f and S-*-AcG3-1282-a-A-18. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbour joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested. The strains matching probe S-*-AcG3-1282-a-A-18 designed in this study are indicated by an asterisk.
B and C. Fluorescence *in situ* hybridization of replicate NPE-amended reactors SCAS1. The two images correspond to the same microscopic field. (B) Cells binding to probe S-*-OTU45-0720-a-A-20. (C) Corresponding views of DAPI stained cells. Photomicrographs were performed at a magnification of 1000×.
Bar = 5 μm applies to all panels.

and 96% similarity with *Sphingomonas terrae*, a polyethylene glycol-utilizing bacteria (Takeuchi *et al.*, 1993) (Fig. 5A).

Fluorescein-labelled probe Sph492 specific for *Sphingomonas* was used in conjunction to cy3-labelled probe ALF1b to target dominant bacteria corresponding to band C in the RNA-derived profile of DGGE (Fig. 5B–D). The Sph492-targeted population represented around 9.5% of all bacteria and significantly less in control reactors (Table 3).

### *Persistence of dominant populations in NPE-amended reactors*

The occurrence of dominant bacterial populations over extended time periods was analysed by PCR-DGGE.

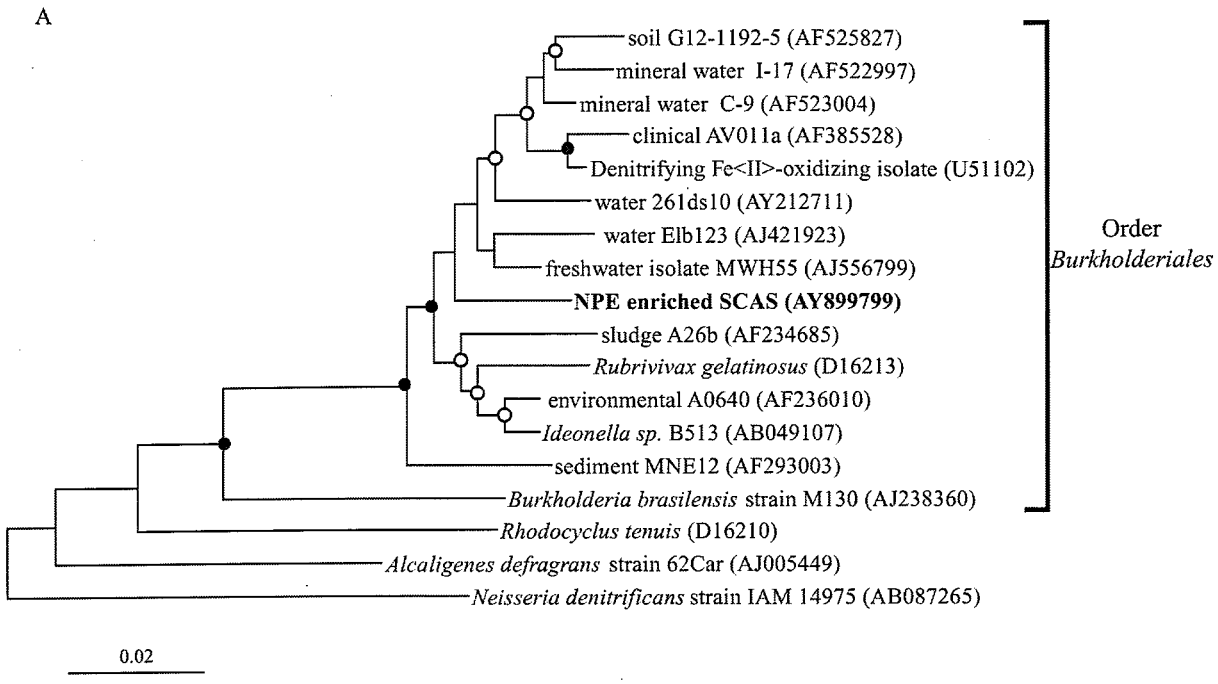


**Fig. 4.** A. Dendrogram showing the phylogenetic position of *Betaproteobacteria* clones obtained from amplification of rcDNA from SCAS1, with primers S-D-Bact-0008-a-S-20 and the reverse complement of *Betaproteobacteria*-specific primer Bet948β. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbour joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested.
B–D. Fluorescence *in situ* hybridization of replicate NPE-amended reactors SCAS1. The three images correspond to the same microscopic field. (B) Cells binding to probe S-*-LARI-0470-a-A-18. (C) Corresponding views of cells binding to probe BET42a. (D) Corresponding views of DAPI-stained cells. Photomicrographs were performed at a magnification of 1000×.
Bar = 5 μm applies to all panels.

Selected bacterial populations, indicated by arrows in Fig. 6, were present in replicate NPE-amended reactors from month 12 and persisted throughout the remainder of the experiment.

## Discussion

Suspended growth systems used in wastewater treatment are complex dynamic mixtures of bacterial populations (Wagner *et al.*, 2002). Several evidences point out to a high degree of diversity that confers stability to bioreactors through functional redundancy. In other words, a highly diverse community would guarantee the presence of several different organisms, which are capable of performing the tasks necessary to allow the maintenance of the system function (Fernandez *et al.*, 1999; Zumstein *et al.*, 2000; LaPara *et al.*, 2002; Briones and Raskin, 2003). Reciprocally, in other occasions maintenance of a high microbial diversity depends on the presence of key functional species filling a specialized niche (Eichner *et al.*, 1999; von Canstein *et al.*, 2002).

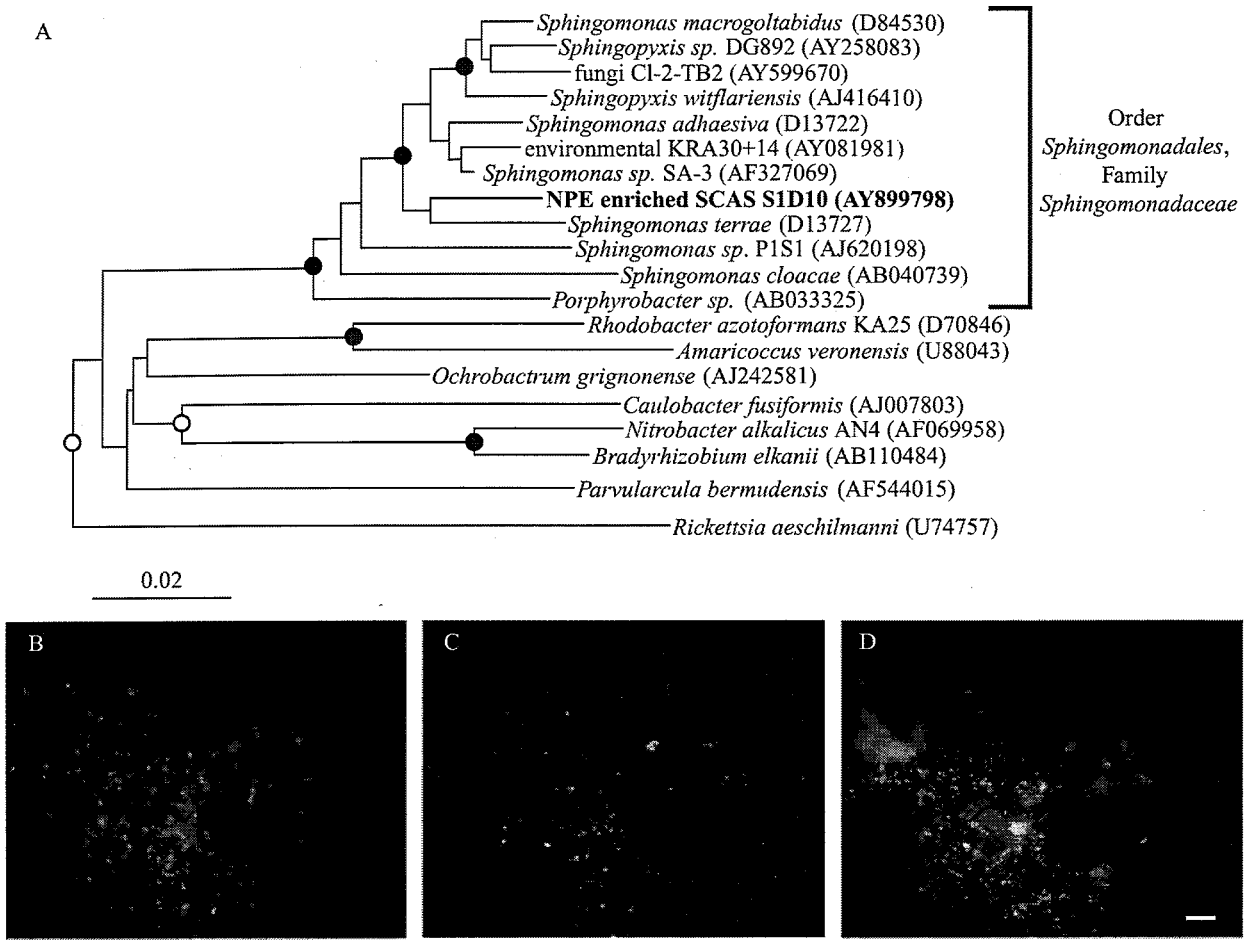


**Fig. 5.** A. Dendrogram showing the phylogenetic position of library clone S1D10, which comigrates and is identical in sequence to band C in Fig. 2. Filled circles correspond to branches conserved for all methods tested (maximum parsimony and neighbour joining with more than 70% bootstrap, and maximum likelihood). Open circles correspond to branches conserved for at least two of the methods tested.
B–D. The three images correspond to the same microscopic field. (B) Cells binding to probe Sph492. (C) Corresponding views of cells binding to probe ALF1b. (D) Corresponding views of DAPI-stained cells. Photomicrographs were performed at a magnification of 1000×.
Bar = 5 μm applies to all panels.

In this study, a combination of SSU ribosomal RNA-based methods of detection was used to analyse the structure of bacterial populations in replicate activated sludge reactors after long-term enrichment with nonylphenol ethoxylate surfactants. Using FISH and quantitative membrane hybridization of total 16S rRNA we had previously shown that the bacterial community of lab-scale activated sludge to which NPE was added in addition to synthetic sewage, was significantly replicated at the broad bacterial group level. Hierarchical (nested) analysis of variance indicated that differences between treatments were statistically significant with regard to probes targeting *Betaproteobacteria*, which increase in NPE-amended reactors, and *Actinobacteria*, which in contrast, were dramatically reduced in treated reactors. Importantly, no significant differences in probe hybridization for any of the probes used were found among replicate reactors (Lozada *et al.*, 2004). However, with these methods it was not possible to observe population shifts at higher resolution, i.e. within the respective bacterial groups (Felske *et al.*, 1997). We have therefore analysed clone libraries and DGGE profiles to monitor the bacterial communities of two treated reactors and two control reactors, aiming at detecting differences and similarities between treatments and between replicates.

In view of the ability of PCR to amplify fragments of low concentration, and the low coverage of the clone libraries, it was expected that sparsely populated OTUs would vary greatly from library to library. Therefore no attempt was made to compare ARDRA types of single OTU between reactors. In contrast to the high eveness observed in clone libraries obtained from control sludge, both NPE-amended reactors were highly dominated by only two related ARDRA types, which accounted for approximately

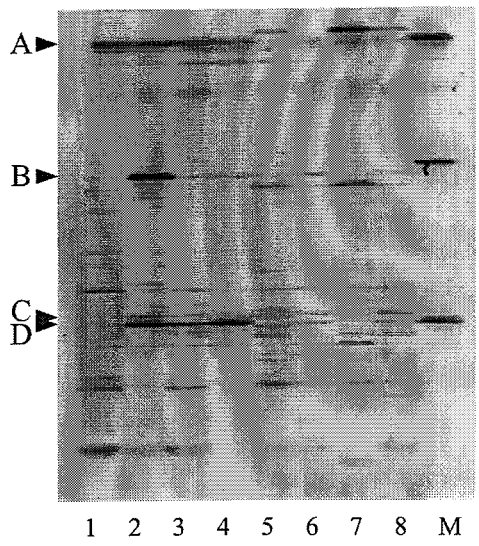


**Fig. 6.** Denaturing gradient gel electrophoresis (DGGE) profile of 16S rDNA fragments obtained from lab-scale reactors fed with (lanes 1–4) or without (lanes 5–8) NPE, at 12 months (lanes 1, 3, 5, 7) and 21 months (lanes 2, 4, 6, 8). Lane M contained a DGGE marker, consisting of a mixture of 16S rDNA fragments. The arrows point to the presence of dominant species in NPE-amended reactors.

30% of the clones analysed. As it has previously been suggested, multiple appearance of the same sequence in 16S rDNA clone library might indicate real abundance (Felske *et al.*, 1997). The opposite is not true, as other codominant sequences in DGGE and FISH experiments were not abundant in clone libraries from NPE-amended reactors.

We have designed new specific probes for quantification of dominant phylotypes in the original communities. When the probes did not work for FISH we have alternatively used real time PCR, a well suited tool for quantification of difficult to culture microorganisms in environmental samples. It is not unprecedented that the linear range of real time PCR in DNA extracts from environmental samples are greatly reduced, compared with standard plasmid DNA, because of inhibitory effects of genomic DNA, loss in fluorescence signal attributed to a large excess of complex DNA, limited probe specificity or a combination of these factors (Harms *et al.*, 2003). However, the quantitative real time PCR data of abundance at the DNA level were in good agreement with the abundance observed in our 16SrDNA clone libraries. In opposition, a most intriguing observation in this study was that despite the abundance of *Gammaproteobacteria* cells, they contain extremely low levels of rRNA. A sharp decrease in ribosome content during late log-phase has been observed before in facultatively oligotrophic marine *Proteobacteria* (Fegatella *et al.*, 1998). Though it is conceivable that low nutrient conditions at the time of sampling may have affected the physiological activity of the *Gammaproteobacteria* cells grown in the reactor, it is uncertain to assert what is the actual physiological state of bacteria within the complex community, as their physiological history and their current physiological state have a dramatic effect on the rate at which detection using rRNA-targeted probe is lost (Oda *et al.*, 2000). Nevertheless, the fact that these cells were detected early within the NPE-amended sludge community and have persisted in replicate reactors over a time that exceeds greatly the mean cellular retention time suggests that these strains have an ecological niche in the managed ecosystems.

By examining both NPE-amended reactors at the rRNA level we were able to determine the phylogenetic affiliation of most *Betaproteobacteria*, previously characterized as one of most abundant groups present in NPE-enriched reactors (Lozada *et al.*, 2004). A specific probe targeting several members of genus *Ideonella*, *Leptothrix*, *Rubrivivax* and other unclassified strains was designed, based on a probe originally developed for the genus *Aquabacterium* (Kalmbach *et al.*, 2000).

Phylogenetic analysis of the sequences from the dominant bands in DGGE of PCR-amplified 16S rDNA V3 and V6–V8 regions showed a clear relationship with the Group III of the highly diverse phylum *Acidobacteria* (Quaiser *et al.*, 2003), within a cluster targeted by probe S-*-OTU45-0720-a-A-20 (Juretschko *et al.*, 2002). Based on the wide ecological distribution and abundance, *Acidobacteria* appear to have an important ecological role. However, only a few members have been described in pure culture, and therefore knowledge about their physiology is still very scarce (Quaiser *et al.*, 2003).

Although no significant differences were observed for *Alphaproteobacteria* using probes targeting broad group of bacteria (Lozada *et al.*, 2004), reverse transcription PCR (RT-PCR) DGGE showed in communities from NPE-amended reactors, the dominance of a band with a sequence related to genus *Sphingomonas*. *In situ* analysis with a *Sphingomonas*-specific probe confirmed the abundance (*c.* 10%) of this phylotype in NPE-amended reactors. The 16S rDNA sequence appeared to be more closely related to *S. terrae*, a polyethylene glycol-utilizing bacteria (Takeuchi *et al.*, 1993), with 96% similarity, than to the nonylphenol-degrading *Sphingomonas cloacae* (Fujii *et al.*, 2001), isolated from a river sediment, recently reclassified as a novel species of genus *Sphingobium* (Fujii *et al.*, 2001; Ushiba *et al.*, 2003), for which 16S rDNA similarity value of only 92% was found.

Despite the fact that a large variety of bacteria species have the ability to grow using NPE as the source of carbon and energy (Barberio *et al.*, 2001), only a few phylotypes are major populations in NPE-amended reactors. Quantitative analysis of these populations showed no significant differences between replicate reactors (Tables 2 and 3).

The replicability of dominant bacterial populations appears to be a characteristic of NPE treatment. The dominance of non-cultivated strains in surfactant-treated reactors found in this study and the fact that bacterial communities are less diverse than in control reactor communities (M. Lozada and L. Erijman, unpublished) can be explained within the framework of the successional theory (Garland *et al.*, 2001). Accordingly, the presence of surfactant in the feeding solution, and possibly the accumulation of the intermediate degradation products in the sludge, have selected for K-strategists, which have adapted to a specialized niche. Dominant populations in surfactant-amended reactors were demonstrated to be relatively stable over time (Fig. 6). Selection of stable dominant populations is in agreement with the expected persistent character of K-strategists. Another factor contributing to the persistence of stable dominant populations is the long solid retention time (SRT) of our systems, selected initially to maximize the growth of slow growing organisms capable of degrading NPE (Lozada *et al.*, 2004). It has been recognized that, because of the non-linear dynamics of complex microbial communities, functional stability of bioreactors increases in parallel with SRT (Curtis *et al.*, 2003).

The feasibility of analysing stable population of yet uncultured microorganisms allows the realization of ecological studies of the organisms that are better adapted to specialized niches. Our findings imply that reproducibility in key populations is attainable for complex suspended growth bioreactor communities working under a selective feeding regime.

## Experimental procedures

### *Laboratory scale sewage plant*

The laboratory SCAS reactors used in this study have been described (Lozada *et al.*, 2004). Shortly, they consisted of four single units containing 3 l of activated sludge treating synthetic sewage, operated in a sequential mode (feeding plus aeration, settling and decanting) at a constant temperature of 20°C. Two of the reactors received additionally 0.1% NPE surfactant, with 10 ethoxy units in average. After 21 months of stable process performance, samples were taken at the end of aeration cycle.

### *DNA extraction and clone libraries construction*

Extraction of DNA from sludge was performed as described (Eichner *et al.*, 1999). Polymerase chain reaction products were produced from genomic DNA by amplification with the bacterial-conserved primer pairs S-D-Bact-0008-a-S-20 and S-*-Univ-1492-a-A-18 (Table 1). The PCR cocktail contained 1.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA), 5% DMSO, 1.25 mM $Mg^{2+}$, 200 nM each primer, 50 μM each deoxynucleotide triphosphate (dNTP) and 1× PCR buffer (Invitrogen). The amplification conditions for PCRs were 94°C for 5 min, 40 cycles of 94°C denaturation for 30 s, 48°C annealing for 1 min, and 1 min extension at 72°C, followed by a 5 min final extension step. Amplified products were ligated into the pGEM T-Easy vector (Promega, Madison, WI) and cloned into competent DH10B *E. coli* cells. Plasmid-containing colonies were randomly selected and 16S rDNA inserts were amplified and subjected to restriction digestion with either HhaI or RsaI (37°C for 4 h) (Vergin *et al.*, 2001). Restriction fragment length polymorphisms were analysed on 3% high-resolution agarose gels, using Gene Tools software (Syngene, Cambridge, UK) and similar patterns were grouped together.

### *RNA extraction and reverse transcription*

All materials and solutions used were RNAse-free. A 800 μl aliquot of samples from sludge was centrifuged, re-suspended in TE buffer (pH 7.5) and transferred to 2 ml screw cap tubes with 0.5 g zirconia silica beads (Biospec Products, OK). A 50 μl aliquot of 10% SDS was added, and the tube was immediately filled with phenol equilibrated in acetate buffer (pH = 5.1). Tubes were transferred to ice, and cells physically disrupted by shaking for 3 min in a reciprocal shaker (Bead-beater, Biospec Products, OK) at maximum speed, at intervals of 30 s. The aqueous phase was transferred to a clean tube and re-extracted twice with chloroform-isoamyl alcohol (24:1). RNA was precipitated with 0.7 volumes of isopropanol and 0.1 volume of ammonium acetate (pH 5.8) and washed with 500 μl of 70% ethanol. The pellet was re-suspended in 50 μl of RNAase-free water.

A total of 200 ng of RNA was used in the reverse transcription reaction, which contained 4 μl of 5× buffer (Promega), 20 μM dNTP mix, 0.6 μM primer S-*-Univ-1392-a-A-15 (Table 1), 200 U reverse transcriptase enzyme (MMLV, Promega) and RNAse-free water to a volume of 20 μl. The reaction was performed as follows: 5 min at 60°C, 1 h at 37°C and 10 min at 72°C. The enzyme was added after the first 10 min at 37°C. Two dilutions of 1/20 and 1/50 of these tubes were used for subsequent PCRs.

### *Denaturing gradient gel electrophoresis*

SSU rRNA genes for DGGE analysis were amplified by nested PCR. One microlitre of the PCR performed as in the preceding paragraph was used as a template for a second round of PCR. Two sets of primers were used in order to amplify a fragment spanning the V3 region (positions 341 and 534 in *E. coli* numbering) and the V6–V8 region (positions 968–1392 of *E. coli*) (Table 1). The forward primers contained a 40 bp GC-rich sequence to prevent complete denaturation during DGGE. The reactions were performed in a total volume of 50 μl. Each PCR contained: 2 ng of DNA template, 3 mM MgCl, 5% dimethyl sulfoxide (DMSO), 0.1 μg $ml^{-1}$ bovine serum albumin, each deoxynucleotide at a final concentration of 0.2 mM, each primer at a final concentration of 0.1 μM and 1.25 U of *Taq* Polymerase (Invitrogen). Amplification reactions were performed under the following conditions: 5 min at 95°C, followed by 30 cycles that consisted in a 95°C denaturation for 45 s, 48°C annealing for 45 s, 72°C extension for 1 min and a final extension of 5 min at 72°C.

Denaturing gradient gel electrophoresis system (CBS scientific, DelMar, CA) was used as specified by the manufacturer. The PCRs were concentrated to 10 µl (Speed Vac Concentrator, Savant) and loaded into gels containing 6% or 8% (w/v) polyacrylamide, depending of the fragment amplified, in 1× TAE [20 mM Tris, 10 mM acetate, 0.5 mM EDTA (pH 7.4)]. The polyacrylamide gels were made with a denaturing gradient ranging from 30% to 70% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis were run at a constant voltage of 50 V (200 bp) and 85 V (400 bp) at 60°C for 18–20 h. After electrophoresis, gels were soaked for 30 min in SYBR green I nucleic acid stain [1:10 000 dilution in TAE (pH = 8.0), Molecular probes, Eugene, OR]. The stained gels were immediately photographed on an UV transillumination Table coupled with a camera module and imaging system (Gene Scan, Hitachi). The positions of the bands on the gel were determined with the aid of Gene Tools software (Syngene). Bands of interest were excised, DNA eluted with an equal volume of TE overnight at 4°C and precipitated with two volumes of ethanol and 100 mM NaCl. The pellet was re-suspended in 25 µl of water. The resulting solution (2 µl) was used as target DNA for a subsequent PCR amplification. The purity and correct running position of each fragment was confirmed by further DGGE.

### *16S rDNA sequencing and phylogenetic analysis*

The 16S rRNA gene (rDNA) templates for DNA sequencing reactions were amplified directly from the respective plasmids with primers M13F and M13R. Amplified DNA was purified by using a Qiaquick PCR cleanup kit (Qiagen, Chatsworth, CA), and DNA concentrations were estimated by gel electrophoresis and ethidium bromide staining. Almost full-length sequences were obtained from five library clones by using primers M13F, M13R and B575F (Table 1) in sequencing reactions. Approximately 500 ng of 16S rDNA was used as the template in dye terminator cycle sequencing reactions. The reactions were resolved on an ABI 3100 DNA Sequencer (Amersham Biosciences, Piscataway, NJ).

Database searches were conducted by using the BLAST program (Altschul *et al.*, 1990) with the GenBank database. 16S rDNA sequences of library clones and DGGE clones were aligned by using the ClustalW software, version 1.7 (Thompson *et al.*, 1994) with sequences retrieved from the database. Alignments were refined by visual inspection. Phylogenetic trees were built by using the maximum likelihood, neighbour joining and maximum parsimony methods (PHYLIP package, version 3.5) and visualized by using Tree View software, version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Outgroups used were *E. coli* (AY319394), *Bacillus* sp. (AB053351) and *Micrococcus luteus* (AY395033).

### *Identification of* Betaproteobacteria

We have followed two independent approaches to assign the members of *Betaproteobacteria* detected with probe BET42a in our previous experiments (Lozada *et al.*, 2004). The first approach was to excise dominant band B from DGGE gels of RT-PCR, stained with SYBR green. The recovered DNA was re-amplified and cloned with the pGEM-T kit. Cloned fragments were checked by DGGE to assess whether the desired band was cloned and were sequenced. The second approach was to use of a taxon-specific primer to amplify and clone a fragment of *c.* 940 bp, using a general bacterial specific primer S-D-Bact-0008-a-S-20 and the reverse complement of *Betaproteobacteria*-specific primer Bet948β (Table 1), which served as the reverse primer, after reverse transcription of rRNA from NPE-amended reactor sludge. Eight of 10 clones that comigrated with band B in DGGE after nested PCR, were sequenced, showing 100% identity.

### *Probe design from clone libraries and DGGE sequences*

UGP-specific probes were designed for the dominant clones in clone libraries and for the major DGGE band D, by using the probe design tool in the ARB software package (Ludwig *et al.*, 2004). Based on comparative analysis of all sequences in the ARB database comprised of publicly available sequences and our in-house clone sequences, the program selected specific regions within the target sequences that allowed their discrimination from all other reference sequences. The selected oligonucleotide target sites were tested for specificity against the 16S rRNA sequences available in the Ribosomal Database Project (RDP) (Cole *et al.*, 2005) by using the probe match analysis. Sequences were subsequently confirmed for specificity using BLAST search (Altschul *et al.*, 1990). Cells not targeted by the probes served as controls of specificity in both real time PCR and FISH experiments. Primers and probe sequences and positions are listed in Table 1.

By modifying a probe designed originally for the genus *Aquabacterium* (Kalmbach *et al.*, 2000), we designed a probe (S-*-LARI-0470-a-A-18) that matched the sequences retrieved from band B in rRNA-derived DGGE and several other members of the order *Burkholderiales*, within genus *Leptothrix*, *Aquabacterium*, *Rubrivivax*, *Ideonella* and other unclassified strains. Probe match analysis within RDP site (http://rdp.cme.msu.edu/probematch/search.jsp) showed that probe S-*-LARI-0470-a-A-18 (Table 1) did not hybridize to any bacteria outside of the order *Burkholderiales*.

### *SYBR green real-time PCR and melting curve analysis*

Two separate real-time PCR assays were developed and optimized to quantify total bacteria and relative abundance of UGP members. Amplification was performed in 25 µl reaction mixtures by using Platinum Taq (Invitrogen), 1× PCR buffer (Invitrogen) in eight-well reaction strips with optical caps (MJ Research). Primers (Table 1) were used for the quantification, at a concentration of 0.2 µM. The efficiency of PCR amplification was checked for various $MgCl_2$ concentrations and annealing temperatures. The optimal amplification conditions for each primer pair were achieved with 3 mM $MgCl_2$. The PCR temperature program was as follows: 50°C for 5 min and 94°C for 10 min, followed by 41 cycles of 92°C for 20 s, 62°C for 1 min and 72°C for 30 s.

The characteristic melting temperature (Tm) of the amplification products, taken immediately after the last reaction cycle, was used to distinguish the products from amplification artefacts that melt at lower temperatures in broader peaks.

The melting curve was visualized with the software Opticon Monitor, version 1.05 (MJ Research).

The specificities of the oligonucleotides were first tested against several genera and mixed bacterial populations by using conventional end-point PCR and gel electrophoresis (not shown). Preliminary testing proved these oligonucleotides to be suited for RTQ-PCR, resulting in the formation of PCR products of the expected size for target DNA and without any PCR product with non-target species tested (data not shown). Therefore, S-*-UGP-1291-a-A-18 was used as reverse primers in conjunction with the f930 bacterial primer to amplify a 395 bp fragment of the 16S rDNA gene and 16S rcDNA (Table 1). General bacterial primer f1114 and universal primer S-*-Univ-1392-a-A-18 (Table 1) were used in parallel to quantify total 16S rDNA copy numbers.

For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or cycle threshold ($C_T$ value). In control reactions without the addition of template DNA, no $C_T$ values were obtained for the UGP-specific assay. Using the total bacterial assay, a $C_T$ value of 25 was obtained, probably because of contamination of PCR reagents, representing a contribution of less than 1.5%, and was subtracted from the data.

The amplification efficiency and linear range of the real-time PCR method were determined by using purified plasmid DNA containing clone S1A3 sequence. The 16S rDNA and rRNA copy numbers were calculated on the basis of the plasmid size (4.5 kb).

The slope of each calibration curve was incorporated into the following equation to determine the reaction efficiency: efficiency = $10^{(-1/\text{slope})} - 1$. The linear range of detection for the real time PCR assay for bacterial 16S rDNA (and rcDNA) was four orders of magnitude, from $1.2 \times 10^4$ copies to $1.2 \times 10^8$ copies of DNA ($n = 4$). The linear range of detection for the real time PCR assay for UGP-specific 16S rDNA (and rcDNA) was also four orders of magnitude, from $1.2 \times 10^3$ copies to $1.2 \times 10^7$ copies of DNA ($n = 4$). The regression coefficient ($r^2$) values for standard curves of real-time PCR assays were 0.996 and 0.995 respectively. For genomic DNA extracted from mixed liquor suspended solids (MLSS) of the four SCAS reactors, consistent results were only obtained at DNA concentration of 2–4 ng per PCR assay, corresponding to $5.5 \times 10^7$ and $1.0 \times 10^6$ copies of DNA. Inhibition was observed at higher DNA concentration. The product of reverse transcription was diluted to contain a number of rcDNA copies comparable to the number of copies of rDNA in Q-PCR reaction. The relative percentage of rDNA and rcDNA molecules of our target was determined by dividing the number of UGP rDNA and rcDNA copies by the total number of bacterial rDNA and rcDNA molecules.

Data were tested for homogeneity of variances and subjected to nested analysis of variance (ANOVA) by applying a model with replicates reactors (SCAS1/SCAS3 and SCAS2/SCAS4) nested within feeding treatment (with NPE/without NPE), using the software package Statistica, version 6 (StatSoft, Tucsa, OK).

### *Fluorescence* in situ *hybridization*

Activated-sludge samples were fixed by the addition of paraformaldehyde to a final concentration of 4% at 4°C for 2 h, and were directly spotted on gelatin-coated cover glass. Dehydration and further application of the oligonucleotide probes were performed according to the procedure previously described (Lozada *et al.*, 2004).

To determine the stringency for optimal probe specificity and signal intensity for probe S-*-LARI-0470-a-A-18, stepwise 5% increments in formamide concentrations from 0 to 40% were used, using a fixed pure culture of *Burkholderia cepacia* (ATCC 10856), containing two mismatches, as the non-target species.

Formamide concentrations used for group-specific probes were: 35% for S-*-OTU45-0720-a-A-20 and S-*-LARI-470-a-A-18, and 20% for *Sph492*. Non-specific binding was checked with probe Non-EUB338 (Wallner *et al.*, 1993). Relative abundance of positive cells for each probe was calculated as percentage of DAPI-stained cells. Data were tested for normality and homogeneity of variances and transformed when necessary. Nested analysis of variance was used to test for significant differences between means of relative abundances comparing treatments (NPE-amended versus control) and replicate reactors within each treatment.

### *Nucleotide sequence accession numbers*

The GenBank accession numbers for the small-subunit sequences are as follows: S3A1, AY294012; S3D1, AY294013; S1A3, AY294014; S1B10, AY382153; S4A5, AY382154; DGGE clone S1b8 AY559028; DGGE clone S3b4 AY559029; DGGE clone S3cl1, AY559030. *Acidobacteria* clone, AY899797; *Sphingomonas* clone, AY899798; *Betaproteobacteria* clone, AY899799; *Gammaproteobacteria* clones AY899800 and AY899801.

## Acknowledgements

The authors thank Alberto R. Kohrnblihtt for allowing us the use of the Engine Opticon system, and Gustavo Gudesblat for his assistance with its operation. We also wish to thank Luis de Tullio for his interest in this project and Thomas M. Jovin for continuous support. Finally, we thank the anonymous reviewers for their valuable comments, which helped us to improve the manuscript. This work was partly funded by FONCyT (PICT 2000 No. 01-09560, PICT 2003 No. 01-14218), CONICET (PIP 2397) and Fundación Antorchas. L.E. is a career member, and M.L. and E.L.M.F. are fellows of CONICET.

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