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Bacterial community shifts in nonylphenol polyethoxylates-enriched activated sludge

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Abstract

A molecular approach was used to evaluate the effect of nonylphenol ethoxylate surfactants on the bacterial diversity in lab-scale activated sludge reactors. Separate bench-scale units were fed synthetic wastewater with and without addition of branched nonylphenol ethoxylates (NP*n*EO). The performance of the reactors, in terms of carbonaceous removal was largely unaffected by the presence of NP10EO in the feeding solution. However, addition of NP10EO exerted a pronounced shift in bacterial community composition. In situ hybridization analyzing larger phylogenetic groups of bacteria with ribosomal RNA-targeted oligonucleotide probes revealed the dominance of clusters composed of *Betaproteobacteria*, accounting for up to one-third of 4′,6-diamidino-2-phenylindol-dihydrochloride (DAPI)-stained cells in NP10EO amended reactors and only 5% of DAPI-stained cells in the controls. These shifts in populations of larger phylogenetic groups were confirmed by dot-blot analysis of rRNA. Members of gamma subclass of Proteobacteria were present in low numbers in all activated sludge samples examined, suggesting that only bacteria affiliated with the beta subclass of Proteobacteria may have a specific role in NP10EO degradation.

Keywords: Nonionic surfactant; Nonylphenol ethoxylates; Activated sludge; Community structure; Fluorescence in situ hybridization; Betaproteobacteria

1. Introduction

Due to their extended range of applications, nonionic surfactants represent a significant contribution to organic pollution in the aquatic environment. Nonylphenol ethoxylates (NP*n*EO) are surfactants widely used in a variety of industrial applications, such as metal and textile processing, paper industry, formulations of pesticides and paints. Commercial NP*n*EO are complex mixtures of isomers and oligomers, as the branched nonyl side chain can take many different structural configurations, with the hydrophylic ethoxy chain (*n*) consisting in typically 3–20 units.

NP*n*EO enter the environment primarily via industrial and municipal wastewater treatment plant (WWTP) effluents (liquid and sludge) [1], and also by direct discharge. Shortening of the glycol chain has been invoked as the main mechanism of biodegradation in pure cultures [2–4] and in river water [5,6]. Recent evidence suggested that central fission of alkylphenol ethoxylate chains might also occur in activated sludge [7]. Biodegradation via ω-oxidation oxyethylene chain

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pathway leads to the formation of nonylphenol ethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC). The widespread presence of these primary degradation products is a cause of environmental concern because of their toxicity. In addition, these products, which are persistent and moderately persistent in groundwater and sediments, have been reported to cause a number of estrogenic responses in a variety of aquatic organisms at low concentrations [8,9].

A number of culturable bacteria with the ability of degrading nonylphenol ethoxylate have been isolated and characterized [4,10]. Although bacterial-mediated conversions of NP*n*EO leading to nonylphenoxy ethoxy acetic and nonyl phenol (NP) and ethylene glycol have not been observed in the laboratory, these degradation intermediates have also been found in receiving waters of sewage treatment plants, sewage sludge, as well as in natural aquatic media. It is therefore conceivable that bacterial populations responsible for degrading NP*n*EO and its metabolites in natural or engineered environments may be different from the strains isolated in the laboratory. In fact, the concentrations of alkylphenol ethoxylated metabolites in effluent from WWTPs varied depending on the plant design and efficiency [11]. Some sewage treatment plants discharge significant amounts of NP*n*EO degradation products in their final effluents and digested sludge compared to the amount of surfactants in wastewater, while others degrade NP*n*EO more or less completely [11]. The underlying causes for this contrasting behavior have not yet been elucidated, especially at the microbiological level.

In this study, laboratory-scale reactors, which have developed a specialized NP*n*EO-degrading microbial community, were investigated using culture-independent methods of analysis based mainly on the 16S rRNA diversity. It is widely recognized that the natural microbial diversity is much greater than the diversity of the bacteria that have been isolated under standard enrichment techniques [12]. The understanding of the microbiological aspects involved in the metabolism of NP*n*EO should contribute to establish the environmental conditions necessary to achieve complete mineralization of degradation intermediates.

2. Materials and methods

2.1. Laboratory-scale sewage plant

The laboratory setup used in this study has been constructed and was operated as a Semi-Continuous Activated Sludge (SCAS) [13]. It consisted of single units containing 3 l of activated sludge freshly obtained from the aeration chamber of a WWTP in Buenos Aires. Air entered at a flow rate of 1.3 l/min. Sterile, 200-fold concentrated synthetic sewage (32 g/l peptone, 22 g/l yeast extract, 6 g/l urea, 1.8 g/l NaCl, 0.8 g/l $CaCl_2 \cdot 2H_2O$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 55.6 g/l K_2HPO_4, 16 g/l KH_2PO_4) and a corresponding amount of dilution water were added daily to the reactors. Two of the reactors received additionally 60 mg/l NP10EO, with 10 ethoxy units (average). Before feeding, air was disconnected for 60 min to allow sludge to settle and 2 l of clarified supernatant left the system via peristaltic pumps. The system was operated at a constant temperature of $20 \pm 1^{\circ}C$. Samples were taken at the end of the aeration cycle to minimize the presence of transient species.

2.1.1. RNA extraction and membrane hybridization

About 800 µl fresh samples from sludge were centrifuged, resuspended 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 of 10% sodium dodecyl sulfate (SDS) was added, and the tube was immediately filled with RNAase-free phenol equilibrated in acetate buffer [pH = 5.1]. Cells were physically disrupted by shaking for 2 min in a bead-beater (Biospec Products, OK) at maximum speed. The aqueous phase was transferred to a clean tube, re-extracted with phenol–chloroform–isoamyl alcohol (25:24:1), and finally with chloroform–isoamyl alcohol (24:1). RNA was precipitated with ammonium acetate (pH 5.8) and washed with 70% ethanol. The pellet was resuspended in 50 µl of RNAase-free water to a final concentration of 1 µg/µl RNA. Extracted RNA was denatured by adding 3 volumes of 2% glutaraldehyde and then diluted to 20 ng/µl in water with poly (A) 1 µg/Ml (Sigma, St. Louis, MO). The 2 µg samples were applied in a total volume of 167 µl to positively charged nylon membranes (Hybond, Amersham Biosciences, Piscataway, NJ) by using a *dot-blot* device (Schleicher and Schuell GmbH, Einbeck, Germany) under slight vacuum. RNA from pure cultures corresponding to each of the bacterial groups tested was used as standards. The DNA oligonucleotide probes used are listed in Table 1. The probes were labeled with [γ^{32}P] ATP by using T4 polynucleotide kinase (Promega, Madison, WI).

Membranes were prehybridized overnight at 40°C in 20 ml hybridization solution, which consisted in 5 × SSC buffer (0.75 M NaCl, 0.075 M sodium citrate, 7% wt/vol SDS, 1 × Denhardt solution and 100 µg/ml salmon sperm DNA in 0.2 M phosphate buffer pH 7.2). Probes were added to each of the membranes and incubated for 18 h at 40°C. Membranes were washed twice for 1 h at the corresponding washing temperature (see Table 2) in 1 × SSC buffer, 1% wt/vol SDS.

Abundances of each group of microorganisms were quantified with a phosphoimager (Storm, Amersham

Table 1
Operating conditions and performance of semicontinuous activated sludge (SCAS) reactors

Parameter	NP10EO		Control	
	SCAS1	SCAS3	SCAS2	SCAS4
COD removal efficiency (%)	85.9±4.3	83.2±3.6	98.3±6.3	94.0±5.5
BOD removal efficiency (%)	96.1±4.0	95.8±5.4	97.2±2.2	98.4±6.2
MLVSS (mg/l)	2030±190	2005±270	2300±160	2410±245
DO (mg/l)	>4	>4	>4	>4
SRT (d)	44	42	50	52
SVI (ml/g SSLM)	16±2	16±4	92±7	80±10

Table 2
Oligonucleotide probes used in FISH and dot-blot experiments and stringent wash conditions

Probe	Specificity	Sequence of probe (5′–3′)	Target[a] site	FA[b] (%)	Temp[c] (°C)	Reference
Eub338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338)	0	49.0	[31]
Alphlb	αProteobacteria	CGTTCGYTCTGAGCCAG	16S (19)	20	45.7	[16]
Bet42a	βProteobacteria	GCCTTCCCACTTCGTTT	23S (1027)	35	40.0	[16]
Gam42A	χProteobacteria	GCCTTCCCACATCGTTT	23S (1027)	35	40.0	[16]
CF319A	CFB phylum	TGGTCCGTGTCTCAGTAC	16S (319)	35	43.7	[23]
HGC69a	Actinobacteria	TATAGTTACCACCGCGT	23S (1901)	25	40.0	[32]
LGC344	Gram-positive bacteria with low G+C content	YSGAAGATTCCCTACTGC	16S (354)	20	40.0	[33]

[a] *E.coli* numbering.
[b] Percentage of formamide (FA) in hybridization buffer.
[c] Temperatures used for hybridization and stringent wash in dot-blot hybridization [34].

Biosciences, Piscataway, NJ). Specific 16S rRNA concentrations were calculated from the blot intensities, using calibration curves prepared with reference series of RNAs extracted from pure cultures. RNA from pure overnight LB cultures of *Agrobacterium tumefaciens*, *Chromobacterium violaceum*, *Acinetobacter johnsonii*, *Myroides odoratus*, *Bacillus subtilis* and *Micrococcus luteus* were used as standard for each of the probes. A 10-fold excess of unlabeled *Betaproteobacteria* probe was added to the labeled *Gammaproteobacteria* probe and vice versa to prevent cross-hybridization. Ribosomal RNA data are expressed as the fraction of the total eubacterial RNA in the sample, determined by hybridization to the Eub338 probe.

2.2. Fluorescence in situ hybridization

Sludge samples were fixed with freshly prepared 4% paraformaldehyde solutions for 3 h at 4°C, except for the HGC69a and LGC probes, targeted at Gram-positive microorganisms, which are not adequately permeabilized under these conditions [14]. For HGC69a and LGC probes, one volume of 100% ethanol was added to one volume of sludge samples and fixed for 4 h at 4°C. Amino-linked oligonucleotide probes (Biosynthesis Inc., Lewisville, TX) were labeled with Cy3 NHS ester (Amersham Biosciences, Piscataway, NJ) following standard protocols [15]. In situ hybridization was performed at 46°C for 90 min with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), and 0.01% SDS), containing 5 ng of each labeled probe/μl [15]. The hybridization stringency was adjusted by adding formamide to the hybridization buffer and NaCl concentration in the washing solution (Table 2). The slides were counterstained with 5 g/ml DAPI (4′,6-diamidino-2-phenylindol-dihydrochloride; Molecular Probes, Eugene, OR). Probes BET42a and GAM42a were used with unlabeled competitor oligonucleotides [16]. The hybridization mixtures were removed and washed at 48°C with washing buffer containing variable concentration of NaCl, 20 mM Tris-HCl (pH 7.4) and 0.01% SDS. Slides were examined with a Leica (Wetzlar, FRG) DM LB epifluorescence microscope using the set of filters A (DAPI), I3 (FITC) and Y3 (cy3). For each sample, more than 1000 cells stained with DAPI were enumerated. The respective concentrations were calculated as the percentage of the DAPI-stained cells. Results were not corrected for nonspecific binding. B&W photomicrographs were obtained with Leica DC250 camera.

2.3. Capillary electrophoresis

Standard solutions were prepared daily by diluting and mixing in the appropriate media stock solutions of the analytes in acetonitrile or water. Technical 4-NP (Fluka, Buchs, Switzerland), 4-NP1EO and 4-NP2EO (Promochem, Wesel, Germany) were used as analyte standards. Acetonitrile (J.T. Baker, NJ, USA) and 2-propanol (Sintorgan, Buenos Aires, Argentina) were HPLC grade solvents; purified water (18 MΩ cm) obtained from a *Simplicity* water purification unit (Millipore, SP, Brazil) was used. SDS, $Na_2HPO_4 \cdot 2H_2O$ O and $NaH_2PO_4 \cdot H_2O$ were p.a. chemicals (Carlo Erba, Milan, Italy). All CE solutions were filtered through 0.45 μm PVDF membrane filters.

A SpectraPHORESIS CE equipment (Thermo Separation Products, CA, USA) consisting of a *VIAL SERVER* autosampler, an *ULTRA* separation module, an *UV*3000 scanning UV/visible detector and a *SN*4000 interface was used; PC1000 software supports the system. CE analyses were performed in a 75-μm inner diameter fused silica capillary of 39.3 cm total length and 33.5 cm length to the detection window. A background electrolyte composition of 49 mM SDS and 40% 2-propanol in a 10 mM phosphate buffer (pH 6.8) was selected. Prior to injection, standards and SCAS supernatant C18 eluates were diluted in a 40% acetonitrile, 5 mM phosphate buffer solution (pH 6.8).

Sample introduction was made in the hydrodynamic mode, for 2.5 s at 0.8 psi; temperature was set at 25°C and the selected run voltage was 20 kV. Detection wavelength was 200 nm. Confirmation of peak assignments for the ethoxylated homologues was carried out by spiking the samples with NP, NP1EO and NP2EO standards.

2.4. Statistical analyses

Nested analysis of variance (ANOVA) was used to compare the bacterial community among reactors. Data were transformed with natural log (ln) where appropriate, and subjected to statistical analyses by applying a model with replicates reactors (SCAS1/SCAS3 and SCAS2/SCAS4) nested within feeding treatment (with NPEO/without NPEO) for each oligonucleotide probe. All analyses were conducted using the software package Statistica, version 6 (StatSoft, Tucsa, OK).

2.5. Aerobic biodegradation of NP10EO

Batch aerobic degradation assays were conducted using 500-ml samples of solutions of NP10EO as the sole source of carbon, at initial concentrations of 400 mg/l as COD, in 1000-ml separation funnels, connected to a supply of air free of oil. Each flask was provided with equal amount of biomass from each of the SCAS (MLVSS = 400 mg/l), micro- and macronutrients and phosphate buffering (pH = 7.4 ± 0.1) at a temperature of 24°C (±2). Degradation was followed by COD analysis at intervals over 48 h.

2.6. Other analytical methods

BOD, COD, DO, MLSS, MLVSS and sludge volumetric index (SVI) were measured in accordance with Standard Methods [17].

3. Results

3.1. Reactors performance

Sludge samples were obtained from four laboratory-scale SCAS maintained on a defined synthetic wastewater for over 1 year. The reactors were run in a fill and draw mode, the volume of settled supernatant equal to the volume of feed added. The settlement period was 60 min, which was sufficient for separation of phases and retention of the biomass. The reactors were not refilled until maximum COD removal from each previous load had ceased. Temperature and pH were kept constant at 20°C in order to exclude shifts in the microbial community composition due to changes in any of these parameters. The carbonaceous material in the feed was oxidized within 6 h after the start of each aeration cycle (not shown). Thereafter, the sludge respires endogenously for the remainder of the aeration period, during which time the only available substrate is NP10EO and its primary degradation products. These conditions, combined with long SRT (over 40 days) were chosen to maximize biochemical oxidation of organics in the activated sludge.

After a stabilization period of 16 weeks, all reactors exhibited a constant performance. The operating performance of the reactors under stable conditions is summarized in Table 1. All systems provided good organic removal capacity throughout the whole experimental period (>96%). The differences between BOD removal and COD removal are explained by the recalcitrant character of NP10EO biodegradation intermediates. Microscopic examination of samples from the reactors revealed significant differences in the structures of the activated sludge flocs (not shown). Accordingly, SVI of NP10EO-treated reactor was consistently lower than controls (Table 1).

Since the abundance of particular metabolites in sewage treatment is very dependent on the treatment conditions [18,19], we have followed the degradation and formation of individual NP10EO oligomers over time during biological treatment. Acclimation of sludge to surfactant was confirmed by the rapid disappearance of foam. A time course experiment shows the fast

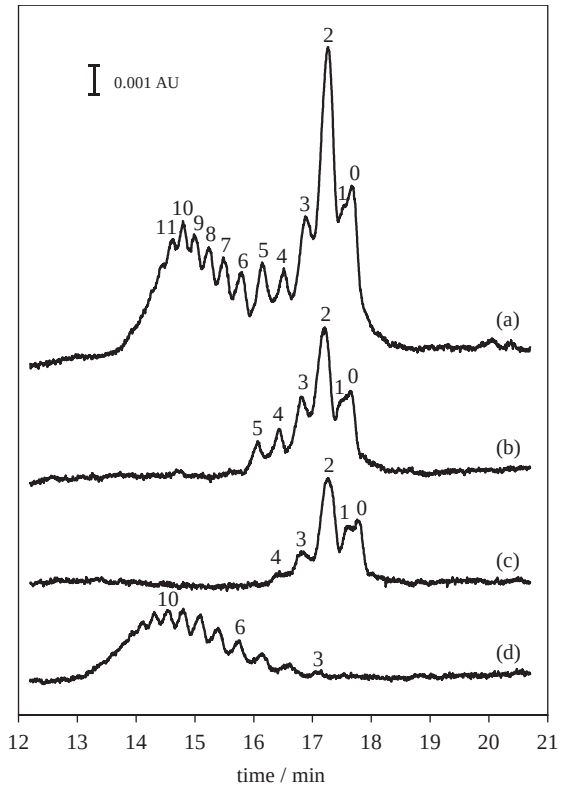

Fig. 1. Electropherograms of SCAS supernatant C18 eluates at different times after feeding with NP10EO: (a) 30 min, (b) 3 h, and (c) 48 h, (d) electropherogram of a 500 μg/ml NP10EO standard solution. The numbers above the peaks indicate the number of ethylene oxide groups (*n*) in the corresponding ethoxylated homologue (NP*n*EO). Experimental conditions were as described in the text.

change in distribution of oligomers with a transient increase in medium-chain oligomers and a larger increase in concentration of short-chain oligomers, including NP (Fig. 1). After 2 h, longer-chain oligomers (NP16–12EO) were no longer detectable. The pattern of short-chain oligomers (NP0–2EO) remained unchanged for the following 48 h. In agreement with previous reports, nonylphenol di-ethoxylate is the major primary metabolite of degradation, with NP1EO and NP3EO as minor metabolites [19–21]. We have not detected production of carboxylic acid analogs of NP10EO.

3.2. *In situ analysis of the activated sludge microbial community enriched with NP10EO*

Analysis of the microbial population structure of the different reactors was obtained using fluorescently labeled group-specific rRNA-directed oligonucleotide probes. Whole cell hybridization with probe Eub338, complementary to a conserved region of the 16S rRNA of the domain *Bacteria* showed that ca. 70% of fixed cells stained with DAPI also emitted probe-conferred fluorescence. Most bacteria that hybridized with probe Eub338 could be detected with the applied group-specific oligonucleotide probes.

Hierarchichal (nested) ANOVA was performed to assess variance components for each probe within replicate reactors and between treatments (with and without NP10EO in the feeding medium). As shown in Table 3, differences between treatments were statistically significant ($p \ll 0.001$) with regard to probes targeting *Betaproteobacteria* and *Actinobacteria*. No significant differences in probe hybridization for any of the probes used were found among duplicate reactors (Table 3). Because of the low variability between replicates, data were pooled to analyze the effect of NP10EO treatment on the bacterial community composition.

Numerically, *Actinobacteria* (Gram-positive bacteria with a high GC content of DNA) dominated activated sludge from unamended SCAS2 and SCAS4 (28% of the DAPI-stained cells) (Figs. 2 and 3a). The next most predominant group corresponded to bacteria belonging to *Alphaproteobacteria* (12%) (Fig. 3a).

A very different pattern represents the microbial communities of the reactors exposed to NP10EO (SCAS1 and SCAS3). In these reactors, sludge composition was dominated by *Betaproteobacteria* (ca. 32% of the DAPI cell number, Figs. 2 and 3a). Cells were bound in densely packed clusters within the bacterial flocs (Fig. 2). The proportion of *Actinobacteria* went down to low levels (ca. 3%). Members of Cytophaga–Flavobacterium–Bacteroides cluster, α and γ subclasses of Proteobacteria and Gram-positive bacteria with low GC content, yielded similar distribution patterns in all types of reactors. The proportion of the gamma-Proteobacteria was below 5% in amended and unamended reactors (Fig. 3a).

3.3. *Surfactant-degradation activity of sludge enriched with NP10EO*

A comparison of the kinetics of surfactant degradation in sludge taken from all reactors was followed up in a static test under aerobic conditions, through the measurements of COD concentrations. The results of the experiment are presented in Fig. 4. Reduction of COD was observed almost immediately by both sludge samples taken from surfactant-enriched reactors, whereas no degradation was detectable after 2 days by control sludge. This different behavior can be attributed to the higher cell density of NPEO degraders in surfactant-treated reactors, proving that the amendment of NPEO resulted in an enrichment of surfactant-degrading population.

Table 3
Nested analyses of variance of the percentage of 16S rRNA hybridization for each oligonucleotide probe

Probe	Variability source	SS	df	MS	F	p
Alphlb	Among replicates within treatment	25.19	2	12.60	0.31	0.74
	Among treatment	7.64	1	7.64	0.19	0.68
	Within replicates	327.34	8	40.92		
Bet42a	Among replicates within treatment	0.32	2	0.16	0.02	0.98
	Among treatment	832.36	1	832.36	109.97	$\ll$0.001
	Within replicates	60.55	8	7.57		
Gam42A	Among replicates within treatment	6.14	2	3.07	0.89	0.45
	Among treatment	0.02	1	0.02	0.01	0.94
	Within replicates	27.65	8	3.46		
CF319A	Among replicates within treatment	2.21	2	1.11	0.27	0.77
	Among treatment	11.20	1	11.20	2.75	0.14
	Within replicates	32.55	8	4.07		
LGC344	Among replicates within treatment	1.91	2	0.96	0.45	0.65
	Among treatment	9.31	1	9.31	4.39	0.07
	Within replicates	16.95	8	2.12		
HGC69a	Among replicates within treatment	0.005	2	0.003	0.38	0.70
	Among treatment	3.17	1	3.17	454.57	$\ll$0.001
	Within replicates	0.056	8	0.007		

3.4. Effect of cultivation on the rRNA composition: membrane hybridization of RNA

Organism abundance was also estimated from the fractional contribution of its specific rRNA molecules to the total ribosome population. Samples of sludge from enriched and control SCAS reactors were subjected to rRNA hybridization analysis. Hybridizations with the group-specific probes were quantified relative to probe Eub338 signal (Fig. 3b). Similar distributions were obtained for all probes used. Additionally, two strains isolated using classical enrichment methods hybridized with the probe for gamma-proteobacteria, further identified as *Pseudomonas putida*. A third strain, positive to the alph1b probe was further identified as *Agrobacterium radiobacter* (not shown).

The effect of cultivation in rich medium on the community structure of activated sludge was also examined (Fig. 3c). In agreement with previous studies [22–24], the results obtained after cultivation were dramatically different from the direct quantification. The rich culture medium favored gamma subclass of Proteobacteria present at low concentration in the original samples and strongly selected against alpha and beta subclass of Proteobacteria and Actinobacteria.

4. Discussion

In this study, we have used a combination of culture-independent methods to analyze the impact of nonionic surfactants, namely NP10EO on activated sludge bacterial community. The experiments were carried out with two replicates per treatment (two control reactors and two test reactors). Nested ANOVA showed that bacterial community composition of replicate activated sludge reactors was not significantly different, and therefore the observed effects were indeed due to the presence of NP10EO in the feeding medium. This result contrast with previous experiments of two laboratory-scale sequencing batch reactors operated under identical conditions, which displayed similar system performance but a divergent prokaryotic community structure [25]. Since the application of broad group-specific probes did not allow one to observe population shifts within the respective bacterial groups, it is likely that higher levels of resolution are needed to observe the dynamic character of microbial community in functionally stable bioreactors [25,26].

Using classical enrichment methods, other authors found that a high diversity of bacteria species have the ability to grow using NP*n*EO as the sole source of carbon and energy [10]. Most of the isolates had been placed within the genera *Acinetobacter*, *Aeromonas*,

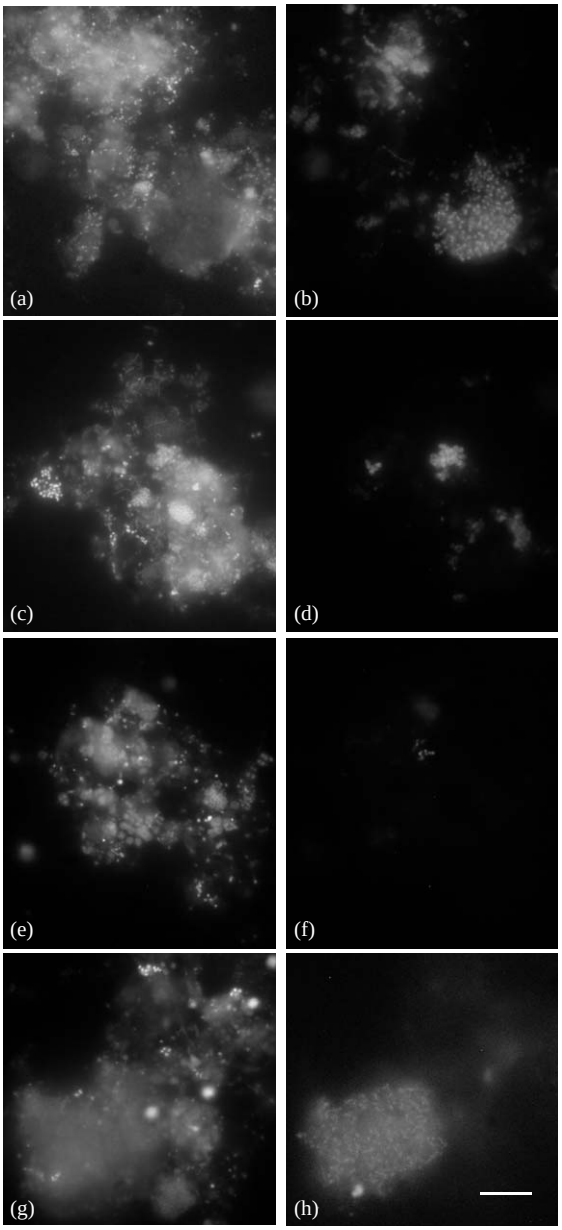

Fig. 2. Fig. 2. FISH of SCAS1 (a–d) and SCAS2 (e–h). Two images are presented for each microscopic field. On the left are DAPI-stained cells. On the right are the corresponding views of cells binding to probes BET42 (b, d) and HGC69a (f, h). All photomicrographs were done at a magnification of 1000 ×. Bar = 10 μm applies to all panels.

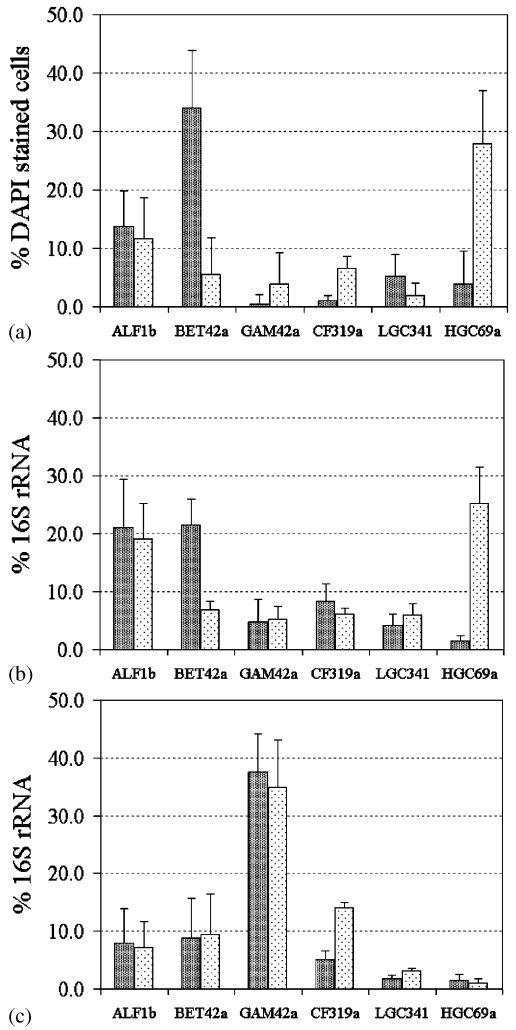

Fig. 3. Comparison of community composition in reactors fed without NP10EO (□) and with 60 mg/l NP10EO (▦), using group-specific 16S rRNA-targeted oligonucleotides as determined by: (a) FISH, (b) dot-blot hybridization of extracted nucleic acids, and (c) dot-blot hybridization of extracted nucleic acids after 48 h incubation in LB. The oligonucleotide probes used and conditions of hybridization are shown in Table 2. Error bars indicate standard error for 2–4 independent experiments.

Shewanella and *Proteus* groups, all belonging to the gamma subclass of the Proteobacteria, the most frequently isolated by culture-dependent methods. Characterization of microorganisms based on pure cultures tend to select for strains with high growth rate under specific in vitro conditions, which are not representative of natural environments. Using dot-blot hybridization and fluorescent in situ hybridization, we showed that strains belonging to gamma subclass of Proteobacteria were present in small proportion in NP10EO amended activated sludge (between 0.4% and 6.8%). We also show that this group becomes predominant upon cultivation in a nutrient-rich medium, confirming that the group abundance observed after cultivation does not represent the original community structure and that the role of these strains in the

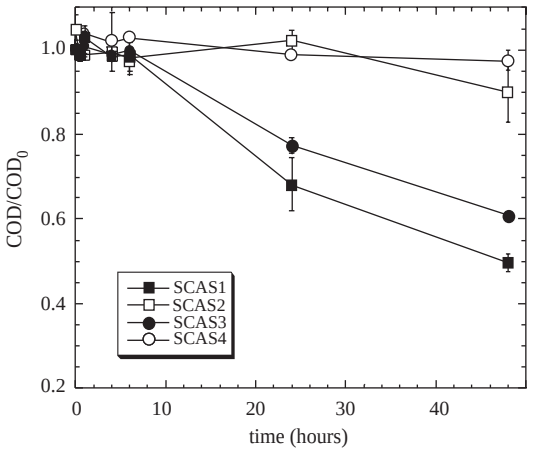

Fig. 4. Aerobic degradation of NP10EO by sludge taken from reactors enriched with surfactant (filled symbols), and by sludge from control reactors (open symbols). Data are presented as mean ± standard error.

degradation of NP*n*EO in natural environments may have been overestimated [22–24]. However, it is conceivable that strains belonging to the beta subclass of Proteobacteria, better adapted to this particular environment, could have acquired the ability to degrade these nonionic surfactants through horizontal transfer, as suggested by Barberio et al. [10].

Quantification of targeted groups by FISH and by dot-blot hybridization gave comparable results, indicating that the incidence of nonspecific labeling to fluorescence signal was minimal. The most noteworthy divergence arises form the less marked predominance of *Betaproteobacteria* observed in dot-blot experiments, compared with FISH quantification. This discrepancy can be rationalized, considering that samples were taken at the end of the aeration cycle, where the activity and therefore the cellular rRNA contents, are lower. Other source of divergence may be originated from the use of bacterial probe Eub338, which does not target some phylogenetic lineages [27].

As seen before in previous activated sludge systems [22,28,29], *Actinobacteria* dominated the activated sludge samples in control samples. Pronounced shifts in the microbial population structure of the activated sludge flocs were observed in NP10EO-enriched sludge. It is conceivable that the reduction in *Actinobacteria* abundance in treated reactors was caused by the sensitivity of this group to the surfactant. *Betaproteobacteria* constitutes the predominant group of bacteria within the NP10EO-amended sludge community. This finding most likely reflects the involvement of this group of bacteria in NP10EO degradation. A numerical domination of *Betaproteobacteria* has been observed in many natural and engineered environments, such as drinking water and activated sludge.

In view of the significant number of cells that did not bind any probe (ca. 30%), we could not exclude the possibilities that undetected fractions of bacteria play an important role in NP10EO degradation. Current research in our laboratory is directed towards the application of the full-cycle rRNA approach [30] that should allow the identification of bacterial key populations enriched in NP*n*EO.

5. Conclusions

1. This study demonstrated that molecular techniques are useful tools in monitoring the effect of NP10EO upon bacterial composition in activated sludge process. Fluorescence in situ hybridization and membrane hybridization results both showed that constant discharge of NP10EO in the wastewater promoted a considerable shift in bacterial community composition of the activated sludge.
2. Statistical analyses of the data established that replicate reactors were not significantly different at the level of broad bacterial probes used in this study.
3. The performance of the reactors, in terms of carbonaceous removal was largely unaffected by the presence of NP10EO in the feeding solution. NP10EO underwent rapid primary degradation with a change in distribution of oligomers, resulting in a transient increase in medium-chain oligomers, followed by accumulation of nonylphenol di-ethoxylate and NP3EO, NP1EO and NP as minor metabolites, which nevertheless were not toxic to bacteria.
4. The composition of the bacterial community structure in the NP10EO fed reactors showed a high proportion of members of the *Betaproteobacteria* grown in the form of clusters, suggesting that this group might have a specific role in NP*n*EO degradation. None of the strains isolated in rich medium hybridized with this probe. These results stress the importance of characterizing the microbial community by means of culture-independent methods.

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