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Nitrate stimulation of indigenous nitrate-reducing, sulfide-oxidising bacterial community in wastewater anaerobic biofilms

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ABSTRACT

The role of the nitrate-reducing, sulfide-oxidising bacteria (NR-SOB) in the nitrate-mediated inhibition of sulfide net production by anaerobic wastewater biofilms was analyzed in two experimental bioreactors, continuously fed with the primary effluent of a wastewater treatment plant, one used as control (BRC) and the other one supplemented with nitrate (BRN). This study integrated information from H_2S and pH microelectrodes, RNA-based molecular techniques, and the time course of biofilm growth and bioreactors water phase. Biofilms were a net source of sulfide for the water phase ($2.01\,\mu mol\,S^{2-}_{tot}\,m^{-2}\,s^{-1}$) in the absence of nitrate dosing. Nitrate addition effectively led to the cessation of sulfide release from biofilms despite which a low rate of net sulfate reduction activity ($0.26\,\mu mol\,S^{2-}_{tot}\,m^{-2}\,s^{-1}$) persisted at a deep layer within the biofilm. Indigenous NR-SOB including *Thiomicrospira denitrificans*, *Arcobacter* sp., and *Thiobacillus denitrificans* were stimulated by nitrate addition resulting in the elimination of most sulfide from the biofilms. Active sulfate reducing bacteria (SRB) represented comparable fractions of total metabolically active bacteria in the libraries obtained from BRN and BRC. However, we detected changes in the taxonomic composition of the SRB community suggesting its adaptation to a higher level of NR-SOB activity in the presence of nitrate.

1. Introduction

Sulfide production is a common problem in sewage environments, due to the high organic input, low dissolved oxygen (DO) concentration and consequent stimulation of metabolic activity of sulfate-reducing bacteria (SRB). Sulfide is involved in deterioration of concrete structures, metal corrosion, toxicity for workers, and foul odors. This has motivated a growing interest in strategies to prevent or diminish sulfide generation. The use of nitrate was proven to be very effective and was originally attributed to inhibition of SRB. Recently, addition of nitrate has been shown to enhance the biological oxidation of sulfide by nitrate-reducing, sulfide-oxidising bacteria (NR-SOB) such as *Thiomicrospira denitrificans*, and

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some strains of *Thiomicrospira* sp., *Thiobacillus* sp., and *Arcobacter* sp. (Gevertz et al., 2000; Kelly and Wood, 2000; Greene et al., 2003; Garcia de Lomas et al., 2006). To date, most of our knowledge on the syntrophic relationship between SRB and NR-SOB has been obtained at laboratory or reactor scale with a few strains isolated from the petroleum industry (Gevertz et al., 2000; Nemati et al., 2001; Greene et al., 2003). Experimental studies involving more complex microbial communities are needed to better understand the SRB/NR-SOB syntrophic relationship and its biotechnological potential in different environments. For this purpose, small-scale bioreactor may allow better experimental control and monitoring than plant-scale studies. Little is known on the SRB/NR-SOB syntrophy in complex biofilms and studies on nitrate inhibition of sulfide production in sewer biofilms are scarce (Santegoeds et al., 1998; Ito et al., 2002; Okabe et al., 2003, 2005).

In this study we test that the nitrate-mediated inhibition of sulfide net production by wastewater anaerobic biofilms occurs via the stimulation of indigenous NR-SOB instead of an inhibition of the sulfate reduction activity. Secondly, we quantify the efficiency of nitrate addition to control sulfide net production in an experimental study carried out for 120 days in two experimental bioreactors, one used as control and the other one supplemented with nitrate. Third, the present work aims to determine how fast the microbial community composition responds to changing conditions caused by nitrate addition. For those purposes, we have integrated information obtained with three different techniques: (i) H_2S and pH vertical microgradients and net sulfate reduction rates (SRR) were measured using microelectrodes; (ii) changes in metabolically active microbial community caused by nitrate addition and those microorganisms involved in sulfide elimination were studied by using RNA-based molecular techniques; and (iii) monitoring the changes occurring in the water phase after nitrate addition. Our results provide information on the process and microorganisms involved in the use of nitrate to control net production of sulfide in anaerobic conditions, and contribute to create an experimental and theoretical framework for future studies directed towards a more efficient use of nitrate at plant scale.

2. Material and methods

2.1. Design and working conditions of experimental bioreactors

Two experimental bioreactors were installed at "Guadalete"-Wastewater Treatment Plant (Guadalete-WWTP) located in Jerez de La Frontera, Spain (see Garcia de Lomas et al., 2006 for further description), and continuously fed with the primary effluent for 120 days (Table 1). The bioreactors consisted of a glass fiber tank (water volume—174 L; biofilm area to wastewater volume, A/V—0.11 cm^{-1}; hydraulic retention time, HRT—3 h) with an inner cylinder made of PVC (diameter—9 cm) placed in the center (Fig. 1) and worked as continuously stirred tank reactors. A submerged pump (ESPA® model Drainex 100MA) continuously pumped the primary effluent from the Guadalete-WWTP to a distribution tank (volume—100 L), from which the sewage was distributed to both reactors with a constant flow of 58 L h^{-1}, using membrane pumps (Dosapro-Milton Roy® model CEGA 90P6P3). Mixing was supplied by motor-induced rotation (Kelvin®, model K200-K90-T4) connected to the central cylinder (thus avoiding bubbling) whose spinning velocity was controlled with a variable speed motor drive AC Tech® (model SL205S) set to 60 rpm. Water level was controlled by an upper drainage and accumulated flocks were withdrawn from the reactors once a week through bottom drainage. Stainless-steel coupons (7 × 3 cm^2) provided by Acerinox S.A., were used as biofilm carriers and incubated inside bioreactors. A set of 6 nylon trays, each one containing 9 coupons, were submerged and attached to the walls of both reactors. Metal coupons were previously degreased in acetone using a soft tissue.

Table 1 – Mean water characteristics for the primary effluent feeding the experimental bioreactors

Variable	Primary effluent
BOD (mg O_2 L^{-1})	207 ± 41 (120); 110–300
COD (mg O_2 L^{-1})	371 ± 61 (120); 231–527
Total solids (mg L^{-1})	1262 ± 192 (120); 932–1790
NO_3^- (mM)	0.016 ± 0.015 (24); 0.000–0.194
NO_2^- (mM)	0.011 ± 0.016 (24); 0.001–0.062
NH_4^+ (mM)	2.11 ± 0.51 (24); 1.07–3.07
pH	7.6 ± 0.1 (120); 7.2–7.9
Dissolved oxygen (%sat)	0.6 ± 0.6 (120); 0.1–3.6
Conductivity (mS cm^{-1})	1.81 ± 0.25 (120); 1.06–2.55
Temperature (°C)	20.8 ± 3.3 (24); 16.2–29.2
Eh (mV)	−424 ± 14 (24); −394/−451
H_2S_{aq} (mM)	2.10 ± 1.02 (24); 0.62–3.13
SO_4^{2-} (mM)	2.02 ± 0.55 (24); 1.19–3.21

These data were obtained from daily measurements determined during the period March–June, 2004. Parentheses indicate the number of samples used for average and SD calculations. In addition, minimum and maximum values registered during the same period are also presented.

Bioreactors were operated for 1 month (pre-operational period) to allow biofilm development before starting nitrate dosing. Bioreactor working conditions were registered by monitoring DO, dissolved hydrogen sulfide (H_2S_{aq}), pH, temperature and Eh. $Ca(NO_3)_2$ as concentrated solution (Nutriox®) was supplemented during 120 days to one bioreactor (BRN) using a membrane pump (Timsa®, model EMP-KKS), to give a final concentration of 0.12 mM NO_3^-. The other bioreactor was used as a control (BRC). Bioreactors were air exposed but set inside a small laboratory in the dark, to avoid photosynthesis and sulfide photooxidation.

2.2. Biofilm biomass and microelectrode measurements

Biofilm biomass was monitored during the experiment by scraping and weighting the biofilm grown on metal coupons ($n = 3$). Vertical profiles of H_2S and pH were measured in biofilms grown inside the BRN and BRC bioreactors for 120 days. Coupons with biofilms, laid on a wet sponge, were carried to the laboratory (30–45 min) in 100% water-saturated

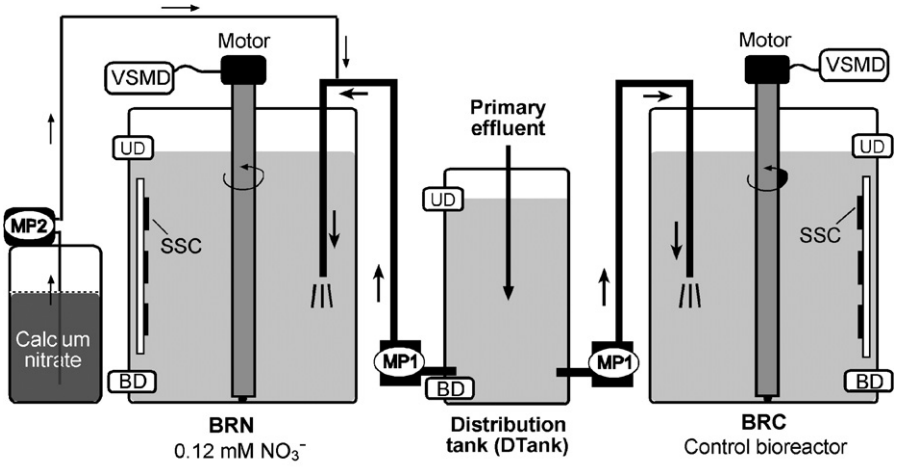

Fig. 1 – Experimental bioreactors setup. Primary effluent was pumped to a distribution tank (*Dtank*), from which was continuously distributed to both reactors with a membrane pump 1 (*MP1*). Nitrate was continuously dosed to one reactor (BRN) at 200 mL h^{-1} (0.12 mM final conc.) with a membrane pump 2 (*MP2*). The other bioreactor was used as a control (BRC). Sewage level in BRN, BRC and *DTank* was controlled with an upper drainage (*UD*), while sludge accumulation was removed by bottom drainage (*BD*). Spinning velocity of the inner cylinder was controlled with a variable speed motor drive (*VSMD*). Nylon trays with stainless steel coupons (*SSC*) were placed along the reactor walls.

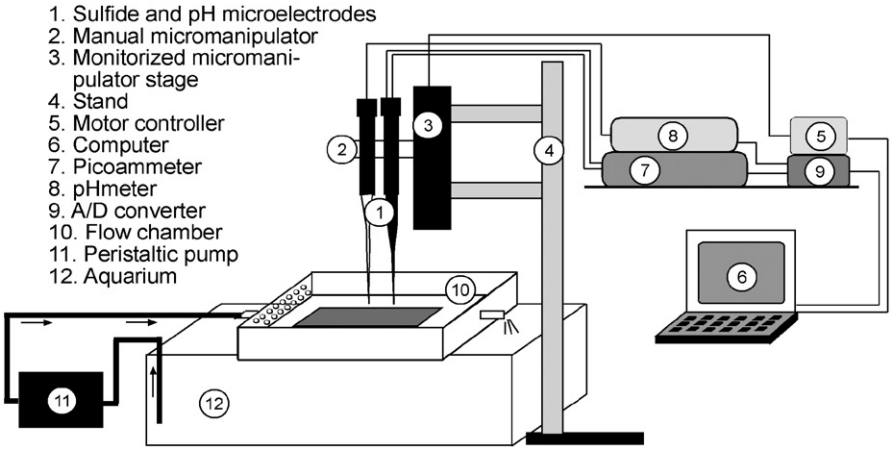

Fig. 2 – Microelectrode setup.

atmosphere using hermetic plastic boxes. Once in the laboratory, the coupons were set in a methacrylate flow chamber (*A*:*V*—0.001 cm^{-1}) under continuous flow (0.2 cm s^{-1}) of wastewater from the corresponding bioreactor (Fig. 2). Biofilms were incubated for at least 2 h before microelectrode measurements to insure steady-state conditions (Satoh et al., 2006). H_2S and pH microelectrodes (Unisense®) were mounted in a manual micromanipulator attached to a monitorized stage connected to a motor controller (Fig. 2). Microelectrodes, with tip diameters of 20–30 μm, were driven into the biofilm with a vertical resolution of 100 μm. H_2S and pH microelectrodes were calibrated and used as previously described (Kühl and Jørgensen, 1992; Corzo et al., 2005). All calibrations and measurements were performed at the same temperature (20 ± 1 °C). Total sulfide concentrations ($S^{2-}_{tot} = H_2S + HS^- + S^{2-}$) were calculated taking into account the measured pH and salinity (Millero et al., 1988), using a $pK = 6.9616$.

2.3. *Flux calculations*

Assuming steady-state profiles, sulfide exportation from the biofilm into the bulk water (J_{up}) was calculated using Fick's first law:

$$J_{up} = -D_{aq}(dC_{DBL}/dz), \quad (1)$$

where D_{aq} is the effective diffusive coefficient of sulfide in the water ($D_{aq} = 8.169 \times 10^{-5}$ cm^{-2} s^{-1}; Stewart, 1998) and dC_{DBL}/dz is the linear sulfide concentration gradient in the diffusive boundary layer (DBL) above the biofilm surface. Net sulphate reduction rates (SRR) within the biofilms were calculated from Eq. (1), but using the effective diffusive coefficient of sulfide in the biofilm ($D_e = 1.39 \times 10^{-5}$ cm^{-2} s^{-1}) instead of D_{aq}, and the steady-state concentration gradient within the biofilm instead of the gradient at the DBL. Porosity was assumed to be 1 and constant with depth within the biofilm (Kühl and Jørgensen, 1992; Ito et al., 2002).

2.4. Water phase monitoring

Physicochemical properties in the bulk water after starting nitrate addition were measured during short-term (first 24 h, sampling frequency = 3 h) and long-term (120 days, sampling frequency = 5 days) monitoring. Samples for NO_3^-, NO_2^-, NH_4^+ and SO_4^{2-} were previously filtered *in situ* through 0.45 μm-pore-size filters and immediately frozen until processed. $NO_3^- + NO_2^-$ were measured with an electrode biosensor (Larsen et al., 2000, Unisense®, Aarhus, Denmark) in the water samples, once hydrogen sulfide was precipitated by adding 0.5 g of zinc acetate (APHA-AWWA-WPFC, 1992). NO_2^- was determined photometrically (Spectroquant® nitrite-test, Merck) and NO_3^- was calculated by difference. NH_4^+ was determined after Koroleff (1969). Samples for H_2S_{aq} were preserved by adding two drops of 0.22 g mL^{-1} zinc acetate immediately after filtration and measured colorimetrically following Cord-Ruwish (1985) method, as described in Garcia de Lomas et al. (2006). Sulfide elimination efficiency, SEE (%) between bioreactors was calculated as follows:

$$SEE(\%) = (H_2S_{BRC} - H_2S_{BRN}) \times 100/H_2S_{BRC}. \quad (2)$$

SO_4^{2-} was analyzed following the standard turbidimetric method and chemical oxygen demand (COD) was determined in homogenized, unfiltered water samples following the colorimetric standard method (APHA-AWWA-WPFC, 1992); pH, Eh and temperature were monitored with portable meters (Crison®).

2.5. Microbial communities

Microbial communities from the water phase and biofilms formed in BRN and BRC were analyzed by RNA-based molecular techniques. Since we are analyzing the bacteria involved in the sulfur cycling in wastewater with and without addition of nitrate, we focused our attention on the SRB and also those capable of oxidizing sulfide, i.e., NR-SOB. By analyzing the metabolically active microorganisms, we were able to greatly simplify the study of the microorganisms involved, since the total diversity in wastewaters is complex as highlighted by previous authors (Curtis et al., 2002).

Samples before nitrate dosing and samples obtained after 3 h, 24 h and 60 days of nitrate addition from both bioreactors (BRC and BRN) were collected and preserved in RNAlater (Ambion, Inc.) on ice until arrival in the laboratory, when they were placed at −80 °C until processed. RNA was extracted using the RNAqueous-4PCR total RNA extraction kit (Ambion, Inc., Austin, TX, USA). Reverse transcription was performed with the enzyme Thermoscript (Invitrogen, Carlsbag, CA, USA) following the manufacturer's recommendations and using the primer 518R (5′-ATT ACC GCG GCT GCT GG; Neefs et al., 1990) for 16S rRNA gene sequences from prokaryotes or the primer DSV838 (5′-SYC CGR CAY CTA GYR TYC ATC; Daly et al., 2000) for SRB. PCR amplifications of resulting cDNAs were carried out with the primer pairs 616F (5′- AGA GTT TGA TYM TGG CTC AG) (Snaidr et al., 1997) and 518R for the total bacterial community and the pair DSV230 (5′-GRG YCY GCG TYY CAT TAG C; Daly et al., 2000) and DSV838 for SRB. PCR thermal cycling conditions were: 95 °C for 2 min and 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min. TaKaRa ExTaq DNA polymerase (TaKaRa Shuzo Co., Otsu, Japan) was used for amplifications in a BioRad iCycler iQ thermal cycler (BioRad, Hercules, CA).

The amplified PCR products from samples obtained after 24 h of treatment were used for constructing 16S rRNA gene libraries. The PCR products were purified using the JetQuick PCR Purification Spin Kit (Genomed, Löhne, Germany) and cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Library screening was performed by denaturing gradient gel electrophoresis (DGGE), as previously described (Gonzalez et al., 2003). Selected plasmids were purified using the JetQuick Plasmid Purification Spin Kit (Genomed, Löhne, Germany) and sequenced by SSAD Sequencing Services (CSIC, Madrid, Spain). A total of 200 sequences were processed. Microbial community fingerprints corresponding to the whole bacterial community or specific to SRB were obtained by DGGE. PCR products for DGGE analyses were obtained from reactions using the primer pairs: 341F-GC (5′-CC TAC GGG AGG CAG CAG; with a GC-rich tail added at its 5′-end) and 518R, following a previously described procedure (Gonzalez and Saiz-Jimenez, 2004).

Sequence data were edited using the software Chromas®, version 1.45. The closest relatives to the microorganisms represented by the retrieved sequences were obtained by homology searches using the Blast algorithm (Altschul et al., 1990) at the NCBI (http://www.ncbi.nlm.nih.gov/blast/). Cloned sequences were screened for chimeric structures using CCODE (Gonzalez et al., 2005).

2.6. Nucleotide sequence accession numbers

Sequence data obtained during this study have been submitted to the NCBI database under the accession numbers DQ409821–DQ410027.

2.7. Statistical analysis

Differences in concentrations of NO_3^-, NO_2^-, NH_4^+, H_2S_{aq}, Eh, pH and COD (mean values) between bioreactors (BRN-BRC) were evaluated by *t*-test, and two-factor ANOVA was used to compare biofilm characteristics (water and organic matter contents, and biofilm biomass) and vertical distributions of sulfide and pH between reactors using Statgraphics® Plus 5.0.

3. Results

3.1. Biofilm growth and bioreactors water phase

Metal coupons were covered homogeneously by biofilms in both reactors during the pre-treatment period (biomass—10–20 mg cm^{-2} after ca. 1 month). Biofilm growth, water and organic matter contents showed no significant differences either during the pre-treatment or after the beginning of nitrate addition between BRN and BRC ($P = 0.0847$, $P = 0.2823$ and $P = 0.0616$, respectively, two-factor Anova). Biofilm reached a maximum biomass of 40–50 mg cm^{-2} and a thickness of 1.5–2 mm (Fig. 3).

Water phase in both bioreactors showed similar physico-chemical properties during pre-treatment period. Both

bioreactors were anoxic and were net producers of sulfide, the sulfide concentration being (ca. 4 mM H_2S_{aq}) higher than that in the input wastewater. The value of pH remained similar to that of input wastewater and nitrate was very low in both bioreactors (0.004 ± 0.004 mM NO_3^-).

Nitrate dosing in BRN induced a rapid and significant ($P = 0.0011$, *t*-test) decrease in sulfide concentrations in the bulk water (Table 2, Fig. 4(a)). SEE reached ca. 82% with respect to BRC within the first 3 h of dosing, increasing to $96.2 \pm 7.3\%$ during long-term monitoring. BRC went on operating as net producer of sulfide (0.7 mM S_{tot}^{2-} h^{-1}). Net balances were obtained from effluent data based on average concentrations during 120 days (Table 2), minus influent data corresponding to the primary effluent (Table 1). Despite the strong decreases in sulfide concentration in BRN, no significant differences were observed in sulfate concentrations in the input wastewater, BRN and BRC (Fig. 4(b)). Continuous nitrate dosing to BRN resulted in a higher nitrate concentration in the bulk water of BRN than in BRC ($P < 0.001$). NO_3^- mean concentrations for BRN and BRC were 0.056 ± 0.067 and 0.004 ± 0.004 mM, respectively (Table 2, Fig. 4(c)). NO_2^- was significantly higher in BRN than in BRC ($P < 0.001$, *t*-test) although the differences tended to disappear towards the end the experiment (Fig. 4(d)). NH_4^+ was the major component of the total inorganic nitrogen (TIN) budget ($TIN = NO^{3-} + NO^{2-} + NH_4^+$), accounting for 96–99% of TIN in BRN and BRC, respectively. NH_4^+ showed no significant differences ($P = 0.6702$, *t*-test) between bioreactors, showing concentration very similar to that in the input wastewater (Fig. 4(e)). Nitrate dosing caused additional changes in other physicochemical variables. Mean redox potential (Eh) and pH were significantly higher in BRN ($P < 0.001$, *t*-test) than in BRC. Eh showed a clear increase during the first few days of nitrate addition but the differences with BRC tended to decrease with time (Fig. 4(g). The pH was 0.20 and 0.45 units higher in BRN than in BRC during the short and long-term assessments (Fig. 4(h). COD measurements showed no significant variations between the reactors ($P = 0.2571$, *t*-test, Fig. 4(f)).

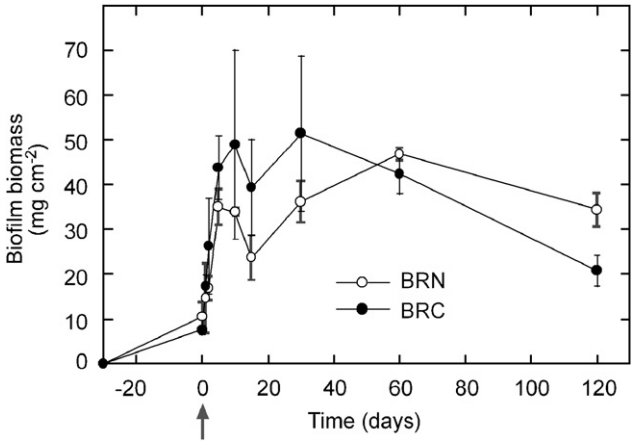

Fig. 3 – Biofilm biomass (grams of fresh weight cm^{-2}) in BRN and BRC during the experiment. Each point represents the mean value for three different coupons ± SD. The gray arrow indicates the starting of nitrate addition.

3.2. *Effect of nitrate on sulfide fluxes in the biofilms*

The measurement of S_{tot}^{2-} at the biofilm–water interface and within biofilms grown in BRC and BRN indicated that BRC-biofilms were a net source of sulfide for the water phase ($J_{up} = 2.01 \pm 0.76$ μmol m^{-2} s^{-1}, $n = 9$), but BRN-biofilms did not export sulfide (Fig. 5). Interestingly, nitrate addition led to cessation of sulfide release to the water phase but did not suppress sulfate reduction activity at the bottom of the biofilm. In presence of nitrate, sulfide production layer moved deeper into the biofilm, from 0.2–0.5 mm in BRC-biofilms to 1.1–1.5 mm in BRN-biofilms, closer to the metal surface (Fig. 5(a)). Microelectrodes data indicated that net SRR inside the biofilms were 0.26 ± 0.09 and 0.70 ± 0.11 μmol S_{tot}^{2-} m^{-2} s^{-1} (average ± SD, $n = 9$), for BRN and BRC respectively. Vertical profiles of pH (Fig. 5(b)) confirmed that the differences found in the bulk water pH between both bioreactors existed within the biofilms as well. The pH was about 0.7 units higher in BRN-biofilms than in BRC-biofilms ($P < 0.0001$, two-factor ANOVA) (Fig. 4(b)). The shape of the vertical microprofiles of

Table 2 – Average values ± SD of different variables measured in the bulk water phase of BRN and BRC, during short-term (first 24 h, $n = 8$) and long-term (120 days, $n = 24$) monitoring

	BRN		BRC	
	First 24 h	120 days	First 24 h	120 days
NO_3^-	0.094 ± 0.046	0.054 ± 0.047	0.003 ± 0.004	0.004 ± 0.004
NO_2^-	0.039 ± 0.024	0.013 ± 0.011	0.005 ± 0.004	0.002 ± 0.002
NH_4^+	1.82 ± 0.36	2.17 ± 0.52	1.91 ± 0.22	2.15 ± 0.47
H_2S_{aq}	1.19 ± 0.49	0.16 ± 0.08	3.76 ± 0.90	4.19 ± 0.93
SO_4^{2-}	2.92 ± 0.36	1.94 ± 0.52	2.77 ± 0.28	1.66 ± 0.66
COD	176 ± 75	184 ± 80	123 ± 23	160 ± 76
Eh	-362 ± 11	-401 ± 27	-407 ± 10	-426 ± 8
pH	8.0 ± 0.1	8.0 ± 0.1	7.8 ± 0.1	7.6 ± 0.1

Concentrations are expressed in mM, except COD (mg O_2 L^{-1}), Eh (mV) and pH. Dissolved oxygen was completely absent in both reactors during the course of experiment.

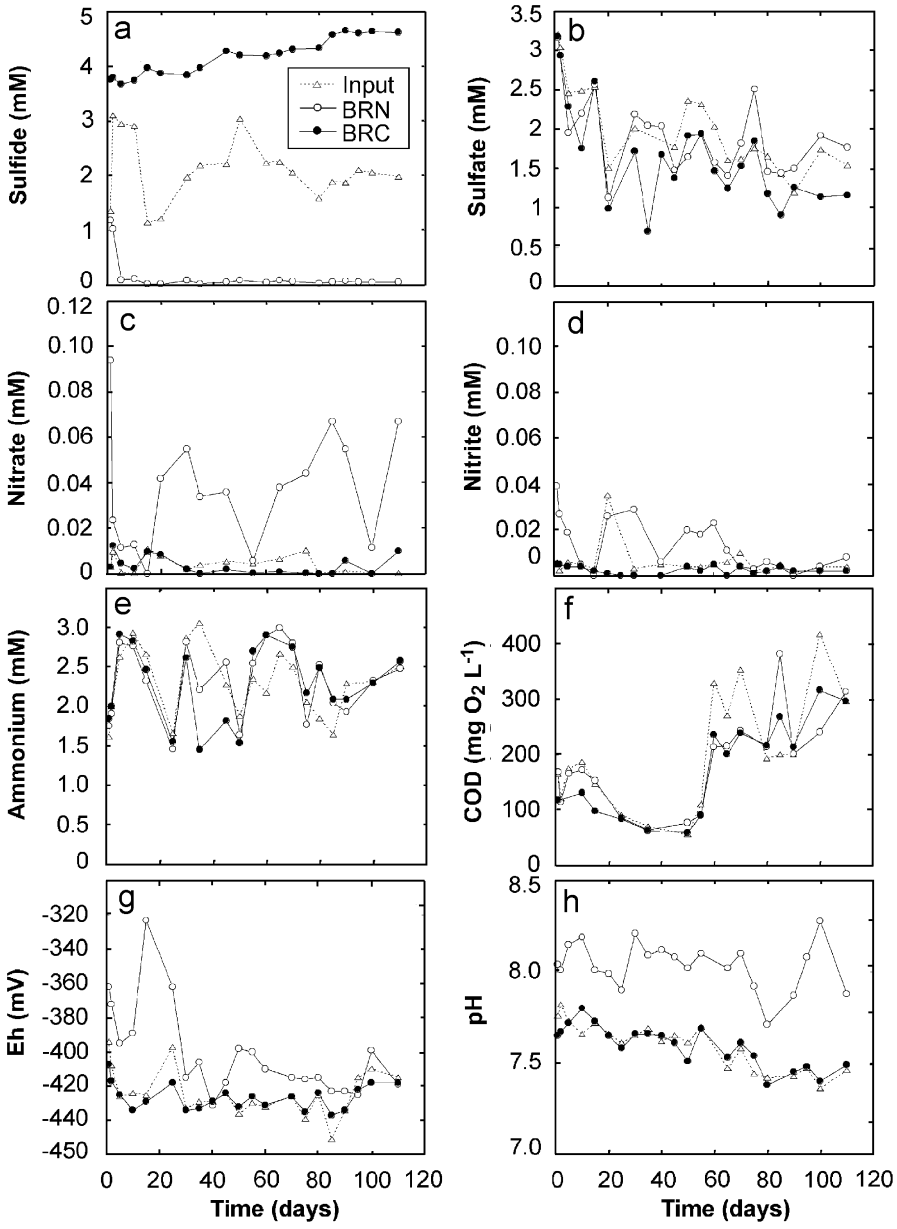

Fig. 4 – Sulfide (a), sulfate (b), nitrate (c), nitrite (d), ammonium (e), chemical oxygen demand (COD) (f), Eh (g) and pH (h) in the input sewage, BRN, and BRC during 120 days. Each point represents the average of duplicate samples. Error bars are not represented for clarity.

pH were slightly different between reactors, showing a higher decrease of pH with depth in BRC-biofilms.

3.3. *Microbial communities in the water phase and in biofilms*

DGGE analyses of 16S rRNA gene products amplified by PCR revealed the presence of a large number of microorganisms in the biofilms and the wastewater phase in both bioreactors (Fig. 6). Cloning libraries obtained from reverse transcription-PCR amplified 16S rRNA gene fragments showed the presence of a significant fraction of SRB in both bioreactors, BRN and BRC. Results showed that the major representative SRB genera in the inflow water, bioreactors water phases and biofilms of both bioreactors were slightly different (Table 3). The fraction of sequences belonging to SRB in the inflow water represented 32.3% of total bacterial community showing metabolic activity (Table 4), but became dominant in the water phase of both BRN and BRC (83.7% and 95%, respectively, of the processed sequences). Using SRB-specific primers for PCR amplification, sequences corresponding to the genus *Desulfovibrio* (40% of sequences) were the most frequently detected SRB in the cloned libraries in the inflow water, followed by the genera *Desulfomicrobium*, *Desulfobacter*, and *Desulfobulbus* (Table 3). In BRN water samples, the most abundant genera of SRB were *Desulfovibrio* constituting a 58% of sequences, followed by *Desulfomicrobium* (34% of sequences). In BRC, the proportion of these genera was

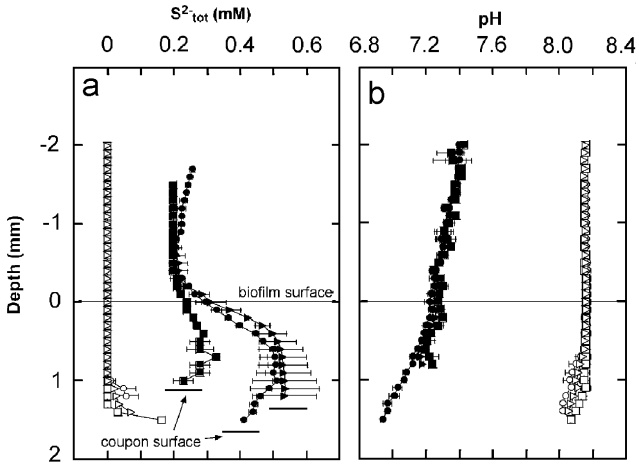

Fig. 5 – Representative sulfide and pH microgradients in three different BRN-biofilms (white symbols) and three different BRC-biofilms (black symbols). Every presented profile is the mean of three profiles in the same coupon ±SD. Note that the biofilm thickness was different: 1.35 mm (○), 1.65 mm (□), 1.55 mm (△), 1.75 mm (●), 1.15 mm (■) and 1.35 mm (▲).

inverted, with *Desulfomicrobium* (50% of sequences) being the most abundant followed by *Desulfovibrio* (36%). Another genus significantly represented among BRN and BRC sequences was *Desulfomonas* (8% and 14%, respectively).

Libraries constructed from BRN-biofilm samples showed sequences belonging to the genus *Desulfomicrobium* (65% of SRB-sequences) as the most frequently encountered SRB, followed by *Desulfobacter* (25%), *Desulfomonas* and *Desulfolobus*. However, in libraries from BRC-biofilms, the sequences belonging to the SRB genera *Desulfobacter* (31%), *Desulfomicrobium* (25%), *Desulfovibrio* and *Desulforegula* (19%) were the major representatives, followed by *Desulfobacterium*. Although the metabolically active SRB communities were different in the BRC- and BRN-biofilms, they represented a similar fraction (around 40%) of the total metabolically active bacteria in the constructed libraries from BRN and BRC (Table 4). However, we found important differences in the proportion of sequences related to the well known NR-SOB (*Thiomicrospira denitrificans, Thiobacillus denitrificans* and *Arcobacter* sp.) obtained from BRC- and BRN-biofilms. Nitrate addition stimulated the NR-SOB community; these sequences represented 19.6% in 16S-rRNA gene libraries from BRN-biofilms but only

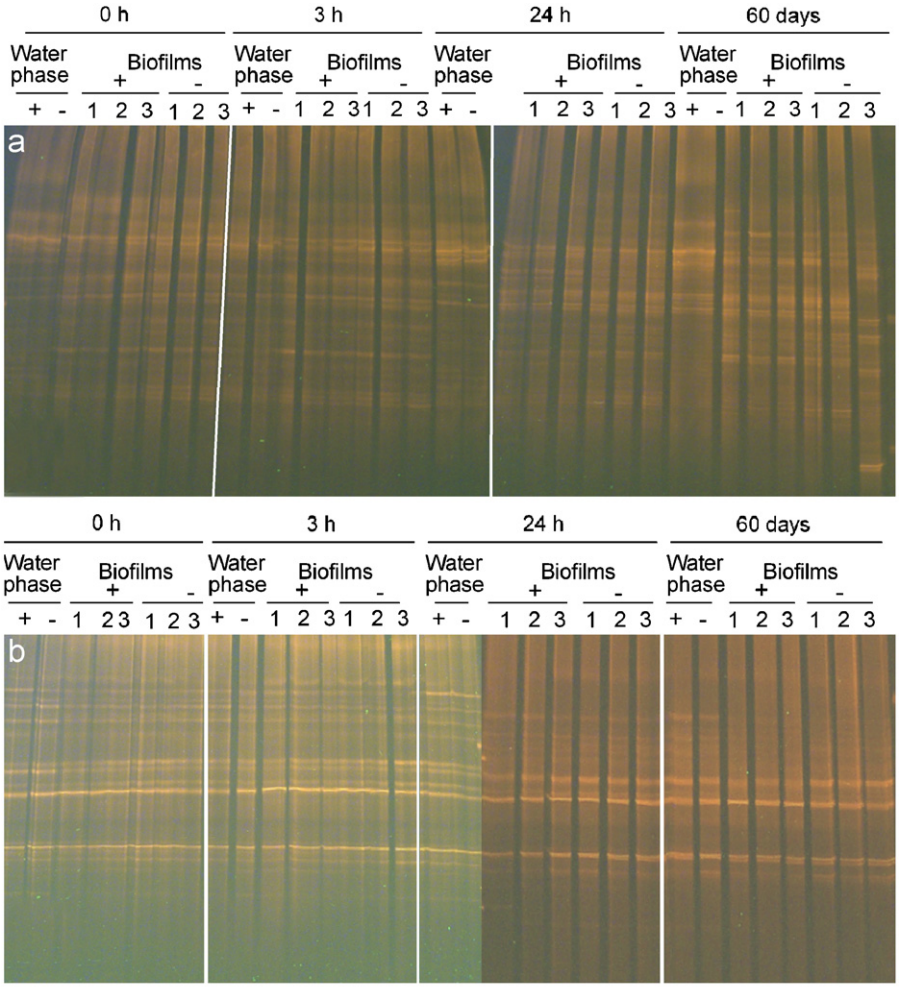

Fig. 6 – DGGE community fingerprints of metabolically active total bacterial community (a) and active SRB (b) detected in the water phase, BRN-biofilms (+), and BRC-biofilms (–), before (0 h) and after nitrate addition to BRN (3 h, 24 h and 60 days).

Table 3 – Percentages of sequences in cloning libraries corresponding to specific genera of sulfate-reducing bacteria (SRB) with respect to total bacterial population in samples of biofilms and the water phase from bioreactors with (BRN) and without (BRC) supplemented nitrate after 24 h

SRB genus	Biofilms		Water phase		
	BRC (%)	BRN (%)	Inflow water (%)	BRC (%)	BRN (%)
Desulfobacter	31	25	10	nd	nd
Desulfomicrobium	25	65	30	50	34
Desulfovibrio	19	nd	40	36	58
Desulforegula	19	nd	nd	nd	nd
Desulfomonas	nd	5	nd	14	8
Desulfobacterium	6	nd	10	nd	nd
Desulfolobus	nd	5	nd	nd	nd
Desulfobulbus	nd	nd	10	nd	nd
Total number of SRB-sequences analyzed	16	20	10	36	38

Sequences from cloning libraries in the inflow water (primary effluent) were also obtained (nd, not detected).

Table 4 – Percentages of sequences (respect to total bacterial population) of metabolically active sulfate-reducing bacteria (SRB) and nitrate-reducing, sulfide-oxidising bacteria (NR-SOB) in BRC- and BRN-biofilms, in the inflow water and water phases of BRC and BRN after 24 of nitrate addition

Bacterial groups	Biofilms		Water phase		
	BRC (%)	BRN (%)	Inflow (%)	BRC (%)	BRN (%)
SRB	40	43.5	32.3	83.7	95
NR-SOB[a]	2.5	19.6	3.2	nd	nd
Total number of sequences analyzed	40	46	31	43	40

nd—not detected.
[a] NR-SOB corresponds to the species related to *Thiomicrospira denitrificans* (an Epsilon-Proteobacteria), *Thiobacillus denitrificans* (a Beta-Proteobacteria) and *Arcobacter* sp.

2.5% of sequences from BRC-biofilms (Table 4). Alpha-, Beta-, Delta-, Gamma-, and Epsilon-Proteobacteria and Bacteroidetes were also detected as metabolically active members of the microbial communities in both BRN and BRC. However, the Verrucomicrobia, Firmicutes and Deferribacteres were found in BRC-biofilms but not in the BRN-biofilms.

4. Discussion

The reactor design, thanks to the cylindrical shape and the central rotating cylinder, assured a well mixed water phase and similar turbulence level at the bioreactors walls where metal coupons were attached. At the same time, undesirable oxygenation was avoided to promote sulfate-reducing activity. Bioreactors were operated with a low HRT and high A/V ratio to maximise the effect of biofilm metabolism on the water phase. DGGE bands corresponding to bacterial communities in the water phase after 60 days were very similar between BRN and BRC, the differences being more striking among theirs biofilms (Fig. 6). Therefore, differences seen in water phase parameters between bioreactors are largely brought about by the biofilm bacteria instead of by planktonic bacteria. Biofilm biomass was not significantly affected by nitrate addition, the growth kinetics being similar in both treatments (Fig. 3). Both bioreactors were anoxic and sulfide concentration before the beginning of nitrate addition to BRN was about two times higher than that in the input water (Table 1 and 2).

Nitrate addition to BRN decreased H_2S_{aq} very quickly (Fig. 4). A SEE of 82% with respect to BRC was reached within the first 3 h of nitrate dosing, increasing up to a mean SEE of 96% for 120 days. The mean SEE reported here are similar to those found by others in different sewer environments with different concentrations of H_2S and added nitrate (Hobson and Yang, 2000; Garcia de Lomas et al., 2006). Based on thermodynamic considerations, nitrate reduction may be assumed to outcompete sulfate reduction due to a larger difference of standard redox potential between the organic electron donors and the inorganic final electron acceptor (Froelich et al., 1979). However, addition of nitrate to sulfate-reducing environments in the oil industry and in wastewater treatment plants was shown to stimulate the activity of NR-SOB rather than inhibit sulfate reduction (Voordouw et al., 1996; Garcia de Lomas et al., 2006). Three independent lines of evidence suggest that sulfate-reduction activity was not fully inhibited by nitrate in BRN. (I) The persistence of sulfate reduction activity, at least in the deepest layer of the

BRN-biofilms, can be deduced from the shape of sulfide profiles (Fig. 5). (II) The existence of active sulfate reducers in BRN-biofilms was confirmed by RNA-based molecular techniques. Interestingly, the proportion of SRB sequences was similar in BRN and BRC (about 40%), suggesting a similar importance of SRB in both bioreactors (Table 3). (III) BRN and BRC operated as net sulfate consumer with respect to the input waste water on long term even when the HRT was relatively low (Tables 1 and 2). In addition, although mean sulfate concentration was slightly lower in BRC than in BRN, the difference was not statistically significant, suggesting a similar level of sulfate reduction. The last statement, although agrees well with the information obtained from molecular biology techniques, does not take into account the probability that sulfate could have been regenerated by the activity of NR-SOB in BRN and likely, at much lower rate, in BRC as well. Mass balance comparison of BRN and BRC for total sulfide and sulfate in the water phase ($H_2S_{aq}+SO_4^{2-}$) revealed important differences between the bioreactors; the values were 2.1 and 5.9 mM for BRN and BRC, respectively. Therefore, it seems that a substantial fraction of the H_2S produced by sulfate reduction was not fully re-oxidised to sulfate in BRN. Elemental sulfur might be produced as intermediary product from sulfide oxidation in our experimental conditions (Okabe et al., 2005; Gadekar et al., 2006).

The added nitrate was used immediately as terminal e^- acceptor by the indigenous NR-SOB community since sulfide was oxidised very quickly after nitrate addition (Table 2, Fig. 4). This quick response suggests that NR-SOB species must be present in BRN before nitrate addition but they were likely limited by nitrate. Mean nitrate concentration in the input water and BRC water phase were very low, 0.016 and 0.004 mM, respectively, compared with the amount of nitrate added to BRN (0.12 mM, final concentration). However, a relatively low level of nitrate seems to be enough to sustain an indigenous NR-SOB community, which was able to quickly respond to a sudden increase in nitrate availability. We detected a small amount of sequences from metabolically active NR-SOB in BRC-biofilms (2.5% of total sequences). Addition of nitrate alleviated limitation of this compound and the metabolically active NR-SOB community increased to represent 19.6% of analyzed sequences in only 24 h (Table 4). In parallel, SEE increased from 82% to 96% and residual nitrate in BRN decreased from 0.094 to 0.056 mM. We do not have information on the changes in the taxonomic composition of microbial community on longer term, but nitrate decreased to a mean concentration of 0.054 mM and SEE increased to 96.7 after 120 days, suggesting a further increase of NR-SOB activity. The development of the NR-SOB community was coincident with changes in the SRB community. Although the percentage of SRB sequences of the total analyzed remained similar in both bioreactors, the relative dominance of several species changed in the BRN-biofilms. This can be interpreted as the adaptation of the SRB community to the higher metabolic activity of the NR-SOB. The syntrophic association between SRB and NR-SOB might be complex. Isolated species of NR-SOB have been shown to inhibit the growth of some SRB species but not of others (Greene et al., 2003; Haveman et al., 2005). Complex successional patterns among SRB and sulfide oxidisers, both aerobic and anaerobic, have been described in wastewater aerobic and microaerophilic biofilms (Ito et al., 2002; Okabe et al., 2005).

The addition of nitrate changed the sulfide balance of the BRN-biofilms. BRC-biofilms were net exporter of sulfide, while all produced sulfide was consumed inside the BRN-biofilms. Addition of nitrate, in aerobic or microaerophilic conditions, to sewer biofilms and to activated sludge immobilised in agar films suppressed sulfide exportation to the overlaying water phase (Okabe et al., 2003). However, this result was mainly explained as the interspecies competition for common carbon sources between heterotrophic nitrate-reducing bacteria and SRB (Okabe et al., 2003). Although aerobic and anaerobic sulfide oxidations were detected, the presence and increases of NR-SOB after nitrate addition was not observed (Okabe et al., 2003). The presence of *Thiomicrospira denitrificans* has been observed in similar conditions more recently (Okabe et al., 2005). Our results demonstrate the role of NR-SOB species such as *Thiomicrospira denitrificans* and *Thiobacillus denitrificans* in the control of sulfide exportation from nitrate-amended anaerobic biofilms (Table 4, Fig. 5). Additionally, we detected changes in the taxonomic composition of SRB community in the BRN-biofilm after 24 h (Table 3). However, no changes in the SRB community were observed in activated sludge immobilised in agar films (Okabe et al., 2003).

Microelectrode measurements indicated that the layer of maximal net sulfate reduction, located close to the biofilm surface in BRC-biofilms (0.2–0.5 mm), moved deeper in the BRN-biofilm (1.1–1.5 mm) after addition of nitrate. Higher nitrate concentration in the water phase of BRN may have allowed nitrate ions to diffuse deeper in the biofilm and increased nitrate uptake by the BRN-biofilms. The higher nitrate availability in BRN-biofilms favored the growth of the NR-SOB community. The work of Okabe et al. (2003) has clearly shown that by increasing nitrate concentration in the water phase one can increase the nitrate penetration depth and the net flux of nitrate into the biofilm. The anaerobic oxidation of sulfide with nitrate as e^- acceptor increased and the layer of maximum SRR seemed to deepen below the nitrate penetration depth. However, sulfate reduction activity might exist in the upper layer of the BRN-biofilm. Microelectrode measurements would not detect this activity if the coupling between SRB and NR-SOB is close enough as to reduce the steady-state concentration of sulfide to undetectable values within the upper layer of the BRN-biofilm. The activity of NR-SOB can regenerate sulfate within the biofilm (Okabe et al., 2005); therefore, the sulfate regeneration rate could support a similar rate of sulfate reduction. Besides, using specific fluorescence *in situ* hybridization probes for some SRB species and for *Thiomicrospira denitrificans*, they were shown to be distributed throughout the biofilm and not just at the bottom (Ito et al., 2002; Okabe et al., 2005). The SRB/NR-SOB syntrophic relationship has theoretical and applied interest. Its biotechnological potential has been confirmed in the petroleum industry and in the biological treatment of wastewater, although it might be relevant to other industrial sectors where sulfide production causes problems as well, such as tanneries, paper mills or beverage industries.

5. Conclusions

- Nitrate addition has been shown to be a very fast and effective strategy to control sulfide generation in wastewater bioreactors having high surface biofilm area to water volume.
- Microelectrodes data indicated that nitrate efficiently suppressed sulfide exportation from biofilms, thus reducing sulfide concentrations in the water phase to negligible levels.
- Sulfate reduction activity was not fully inhibited by nitrate addition. Significant sulfate reduction activity could be detected at the bottom of nitrate-amended biofilms. However, the fraction calculated from the S^{2-}_{tot} steady state profiles could be an underestimate because microelectrodes cannot detect closely coupled sulfate reduction and sulfide oxidation rates.
- RNA-based molecular techniques showed that metabolically active SRB represented a similar fraction of total metabolically active bacteria in the constructed libraries from both BRN and BRC. However, the taxonomic composition of SRB revealed significant changes in microbial community under nitrate dosing.
- The characterization of the metabolically active microbial community using RNA-based molecular techniques revealed that the nitrate-reducing, sulfide-oxidising *Thiomicrospira denitrificans*-related bacteria were stimulated by nitrate addition, resulting in the elimination of most sulfide from the biofilms.

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