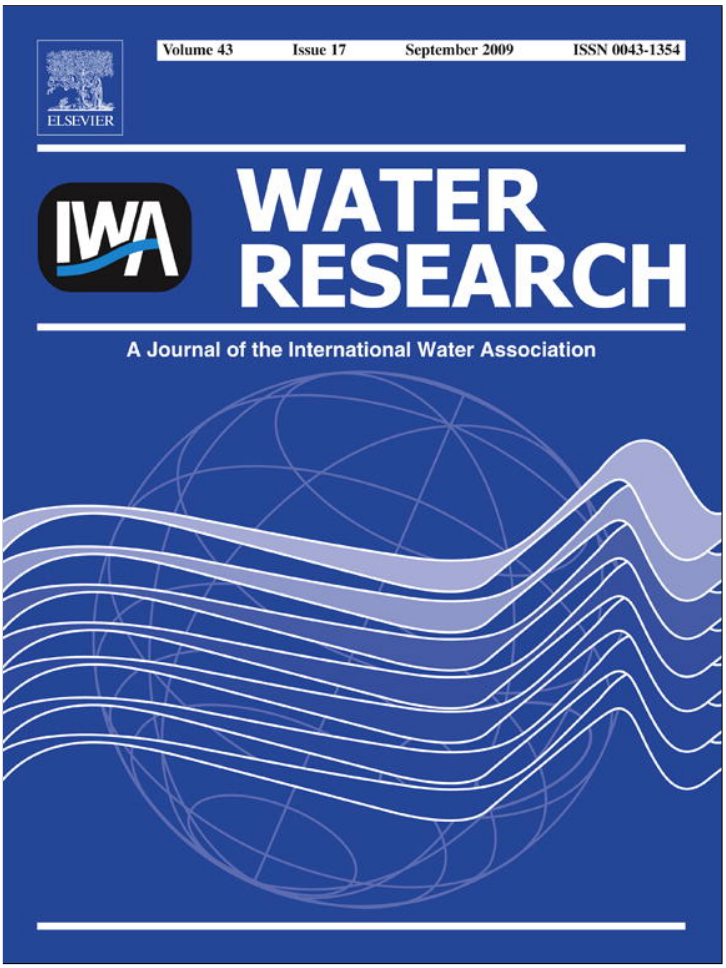

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Impact of nitrate addition on biofilm properties and activities in rising main sewers

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ABSTRACT

Anaerobic sewer biofilm is a composite of many different microbial populations, including sulfate reducing bacteria (SRB), methanogens and heterotrophic bacteria. Nitrate addition to sewers in an attempt to control hydrogen sulfide concentrations affects the behaviour of these populations, which in turn impacts on wastewater characteristics. Experiments were carried out on a laboratory reactor system simulating a rising main to determine the impact of nitrate addition on the microbial activities of anaerobic sewer biofilm. Nitrate was added to the start of the rising main during sewage pump cycles at a concentration of 30 mg-N L^{-1} for over 5 months. While it reduced sulfide levels at the outlet of the system by 66%, nitrate was not toxic or inhibitory to SRB activity and did not affect the dominant SRB populations in the biofilm. Long-term nitrate addition in fact stimulated additional SRB activity in downstream biofilm. Nitrate addition also stimulated the activity of nitrate reducing, sulfide oxidizing bacteria that appeared to be primarily responsible for the prevention of sulfide build up in the wastewater in the presence of nitrate. A short adaptation period of three to four nitrate exposure events (approximately 10 h) was required to stimulate biological sulfide oxidation, beyond which no sulfide accumulation was observed under anoxic conditions. Nitrate addition effectively controlled methane concentrations in the wastewater. The nitrate uptake rate of the biofilm increased with repeated exposure to nitrate, which in turn increased the consumption of biodegradable COD in the wastewater. These results provide a comprehensive understanding of the impact of nitrate addition on wastewater composition and sewer biofilm microbial activities, which will facilitate optimization of nitrate dosing for effective sulfide control in rising main sewers.

1. Introduction

The presence of anaerobic conditions in sewer systems results in significant production of sulfide by sulfate reducing bacteria (SRB) present in the biofilm and solids sediment phases. Release of hydrogen sulfide from the liquid to the gas phase causes several detrimental effects including sewer corrosion, odour nuisance and health hazards. A number of operational strategies have been employed by the wastewater industry to minimize sulfide production in sewers. These range from simple

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Nomenclature

COD	chemical oxygen demand
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
HRT	hydraulic retention time
IC	ion chromatography
NR-SOB	nitrate reducing, sulfide oxidizing bacteria
PCR	polymerase chain reaction
SAOB	sulfide anti-oxidant buffer
SE	standard error
SRB	sulfate reducing bacteria
VFA	volatile fatty acids

strategies like mechanical cleaning, to strategies that involve the addition of one or a combination of chemicals, such as nitrate, oxygen, metal salts (Fe^{2+}, Fe^{3+}, Zn^{2+}), alkali, chlorine, ozone or hydrogen peroxide, to more sophisticated strategies like the addition of SRB metabolic inhibitors or biocides (Boon, 1995; Boon et al., 1998).

Addition of a thermodynamically favourable electron acceptor like nitrate has been used over the last 70 years to control odours and sulfide production in many environments, including sewage systems and oil reservoirs (Bentzen et al., 1995; Heukelekian, 1943; Hubert and Voordouw, 2007; Jenneman et al., 1986; Mathioudakis et al., 2006; Zhang et al., 2008). Different nitrate salts (e.g. sodium and calcium nitrate) and different dosing concentrations have been trialled. Nitrate additions of 10 mg-N L^{-1} to wastewater successfully decreased sulfide concentrations from 4.2 mg L^{-1} to 0.2 mg L^{-1} in a 2.4 km long rising main (Bentzen et al., 1995). Saracevic et al. (2006) applied 40 mg-N L^{-1} nitrate to a 5.0 km long rising main sewer, and discovered that after a lag time of 3–4 days, nitrate reduced sulfide concentrations from 10–20 mg-S L^{-1} to below 2–3;mg-S L^{-1}. Nitrate concentrations of 5 mg-N L^{-1} in wastewater were reported to be sufficient to inhibit sulfide production in a 61 km long gravity sewer (Rodriguez-Gomez et al., 2005).

While there is conclusive evidence showing the effectiveness of nitrate addition on sulfide control, a full understanding of the mechanisms involved is still missing. A number of mechanisms have been described as potentially contributing to the inhibitory effects of nitrate: (i) nitrate addition can increase the redox potential of wastewaters and thus decrease sulfide production by SRB (Allen, 1949; Poduska and Anderson, 1981); (ii) prolonged inhibition has been attributed to the cytotoxic effect of products like nitrite (Eckford and Fedorak, 2004) or nitrous oxide (Jenneman et al., 1986), formed during nitrate reduction, on SRB metabolism; (iii) SRB activity is hindered by competition with nitrate reducing bacteria for organic electron donors (Hubert and Voordouw, 2007); and (iv) nitrate addition increases the pH of the wastewater due to denitrification (Rust et al., 2000), which in turn decreases sulfide production and stripping to the gas phase.

The goal of this study is to gain further understanding of the impacts of nitrate addition on wastewater composition and sewer biofilm microbial activities. In particular, the research aims to answer the following research questions:

1. Does nitrate addition inhibit the activity of SRB in sewer biofilm?
2. How does nitrate achieve effective sulfide control in sewers? Does nitrate addition stimulate sulfide oxidation coupled with nitrate reduction? Evidence has been produced in the last few years to demonstrate that nitrate reducing, sulfide oxidizing bacteria (NR-SOB) proliferate when nitrate is added to anaerobic wastewater containing sulfide (De Gusseme et al., 2009; Garcia-de-Lomas et al., 2007). If this process occurs in sewer conditions, it could play a significant role in sulfide control.
3. What is the impact of nitrate addition on methane concentrations in sewers? Substantial methane production has been recently reported in rising main sewers (Guisasola et al., 2008). Methane is a potent greenhouse gas (IPCC, 2006) and is explosive in confined spaces (Spencer et al., 2006). Methanogens, like SRB, thrive under anaerobic conditions, and their activity could potentially be inhibited by nitrate. The addition of nitrate for sulfide control may therefore have an additional beneficial environmental effect in controlling methane discharge from sewers.

Two laboratory scale sewer systems mimicking rising mains were used in this study. Both systems were fed with real sewage, with the experimental line also receiving nitrate. The research aims stated above were addressed by comparing the long-term performance of the two lines, measuring the activities of sewer biofilm in batch tests under various conditions, measuring the microscale distribution of sulfide and nitrate within the biofilm in conjunction with select batch tests, and by analyzing microbial composition using denaturing gradient gel electrophoresis (DGGE). Knowledge was gained through the integrated use of all information obtained.

2. Materials and methods

2.1. *Laboratory reactor experimental set-up and operation*

Two parallel laboratory scale rising main sewer systems (Fig. 1a), each consisting of four reactors (named RM1–4) connected in series, were operated in a temperature controlled laboratory (20 ± 1 °C). One system was used as the experimental line, while the other was maintained as a reference line. The inner diameter and volume of each cylindrical reactor was 80 mm and 0.75 L respectively. Biofilm was grown on the walls of the reactors, and on plastic Kaldnes carriers (circular, 1 cm diameter; Anox Kaldnes, Norway) placed on rods inside the reactors (Fig. 1b). The carriers facilitated intact biofilm removal for microscale and molecular studies. Further details of the reactor design and properties are available in Gutierrez et al. (2008). Mixing in the reactor was provided by the pump flow and by the magnetic stirrer, the contribution of the latter being dominant. The stirring conditions in the reactor corresponded to a Reynolds number of 6400 indicating a turbulent flow regime.

Both lines were fed intermittently with real sewage through a peristaltic pump (Masterflex Model 7520-47). Operation of the pump was programmed to mimic that of the UC09 rising main, which is a 1.1 km rising main located at the Gold Coast,

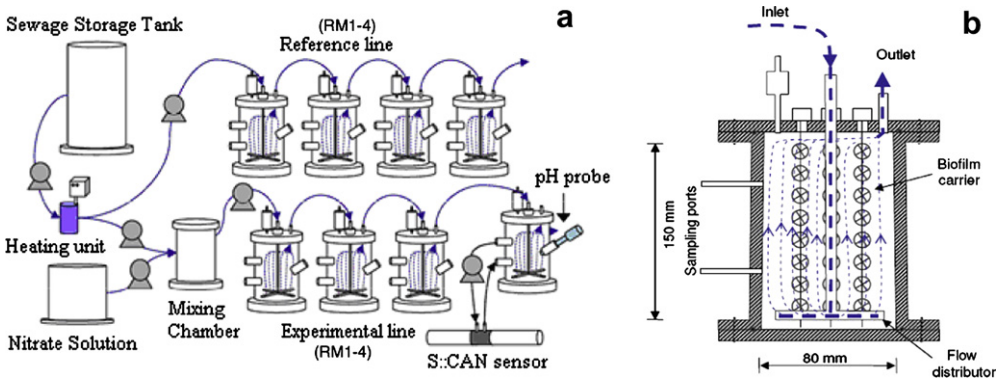

Fig. 1 – Schematic of the (a) laboratory scale rising main sewer systems, and (b) sectional view of a reactor.

Australia. A daily total of 16 pump cycles were maintained with a diurnal variation of sewage hydraulic retention time (HRT) between 2 and 10.5 h. The pump was turned on for 2 min every cycle. When the pump was turned off, the flow rate immediately dropped to zero and quiescent conditions were maintained similar to that in real rising mains. Fresh sewage was collected weekly from a wet well at Robertson Park, Indooroopilly (Brisbane, Australia), and transported immediately to the laboratory. The collection point is in a predominantly domestic catchment and collects sewage flows from gravity sewers in the surrounding local area. The wastewater composition changed from batch to batch and showed large variations in sulfate, volatile fatty acids (VFA) and chemical oxygen demand (COD) concentrations. The wastewater contained sulfate, sulfide, methane and VFA at concentrations of 10–25 mg-S L^{-1}, <3 mg-S L^{-1}, <5 mg-COD L^{-1} and 50–100 mg-COD L^{-1}, respectively. The sewage was stored at 4 °C to minimize biological transformations during storage and heated to room temperature (20 °C) before being pumped into the reactors.

The lines were operated for several months to establish pseudo steady-state conditions and to establish mature anaerobic biofilm on the reactor walls and carriers (phase 1). Analysis of microbial activity in the liquid phase showed that the suspended biomass had a negligible (<2%) contribution to the sulfide production in the reactor (Gutierrez et al., 2009). Sulfide production was therefore attributed to biofilm activities. The measured sulfide production rate per area of biofilm was approximately 2.0 g S m^{-2} day^{-1} in both lines, and the trends in sulfide concentrations over HRT were similar to UC09 (Sharma et al., 2008). Nitrate was thereafter dosed in the experimental line for a period of 155 days (phase 2). Day 0 represents the start of nitrate dosing in the experimental line. Nitrate was added from a stock solution into RM1 during the period of sewage pumping and the nitrate flow was adjusted to maintain an initial nitrate concentration of 30 mg-N L^{-1} in the freshly pumped sewage. The nitrate dosing concentration was identified from communications with local wastewater authorities (Gold Coast Water and Sydney Water Corporation) as being reflective of typical nitrate concentrations obtained in real rising mains after dosing. No changes were made to the routine operation of the reference line in phase 2. Detailed tests were conducted in both phases to investigate the impact of nitrate on microbial biofilm activities and composition.

2.2. *Performance monitoring of the two lines*

The long-term effect of nitrate addition on sulfide control was evaluated by 24 h monitoring of sulfide and nitrate concentrations in RM4 in both the experimental and reference lines towards the end of phase 2 using the S::CAN UV-VIS spectro::lyser (Messtechnik GmbH, Austria). The methods for these measurements have been described in Sutherland-Stacey et al. (2008). The sensor was attached to the outlets of these lines as shown in Fig. 1a. The dissolved sulfide and nitrate concentrations were recorded online every 2 min for five 24 h periods. Sulfide and nitrate readings from the S::CAN sensors were verified by drawing random liquid samples from the outlets for chemical analysis using ion chromatography (IC).

The extent of carbon consumption due to anoxic conditions was also examined in these lines. Effluents from both sewers were collected in two separate tanks over 24 h. Five samples were then taken from these tanks and analyzed for VFA, soluble COD and total COD. These parameters were also measured in the inlet sewage. The impact of anoxic conditions on methanogenesis was assessed by drawing samples from the outlets of the reference and experimental lines under routine operational conditions and analyzing for the presence of dissolved methane. All analytical methods are described in Section 2.4.

2.3. *Batch tests*

Batch experiments were carried out in the experimental line to investigate in detail the impact of nitrate addition on microbial biofilm activities. The purposes and experimental conditions of these tests are summarized in Table 1. The continuous operation of the system was temporarily halted for the duration of each of the batch tests. The reactor was then filled with wastewater specific to the study. A dissolved oxygen (DO) probe fitted in the reactor was used to confirm that DO was below 0.1 mg-O_2 L^{-1}. During each test, liquid samples were drawn at selected intervals from a sampling port fitted with Tygon® tubing 06419-16 into a 5 mL plastic syringe. The samples were drawn carefully so as to minimize the chances of sulfide stripping and/or oxidation. They were immediately filtered using 0.22 μm filters (Millipore, Millex GP) into vials containing preservative sulfide anti-oxidant buffer (SAOB; Gutierrez et al.,

Table 1 – Summary of batch tests in the experimental line.

Objective of studies	No. of studies	Reactor	Wastewater conditions	Sampling intervals	Operational conditions	Analyses
To determine the long-term effects of nitrate on the sulfate reduction capabilities of the biofilm	4 (phase 1), 13 (phase 2)	RM1-4	Fresh sewage	30 min (up to 2 h) + 1 h thereafter (up to 3 h)	Stirring (240 rpm)	Dissolved S, VFA, methane
To determine the short-term effects of nitrate on SRB activity/adaptation of biofilm to nitrate	7 (phase 1)	RM1	Fresh sewage + nitrate added (10–40 mg-N L^{-1})	30 min (up to 3 h)	Stirring (240 rpm)	Dissolved S, dissolved N, methane
To determine the nitrate reduction capabilities of the biofilm	5 (phase 2)					
To investigate combined biological and chemical sulfide oxidation with nitrate	5 (towards end of phase 2)	RM1	Sewage with high sulfide (19.8 ± 2.1 mg-S L^{-1}) and low sulfate (8.6 ± 1.8 mg-S L^{-1}) + high nitrate added (48.4 ± 1.4 mg-N L^{-1})	12 min (up to 50 min)	Stirring (240 rpm)	Dissolved S, dissolved N
To investigate exclusive chemical sulfide oxidation with nitrate	1 (phase 2)	Biofilm-free reactor	0.22 μm filtered wastewater, sparged with nitrogen + sulfide added (mg-S L^{-1}) + nitrate added (14.7 mg-N L^{-1})	15 min (up to 1 h)	Stirring (240 rpm)	Dissolved S, dissolved N

2008) for the analysis of dissolved sulfur (S) species (method described in Section 2.4), and into vials without buffer for the analysis of dissolved nitrogen (N) species and VFA (methods described in Section 2.4). Liquid samples were also regularly filtered into freshly vacuumed BD Vacutainer® tubes using a hypodermic needle and analyzed for methane as described in Section 2.4. Rates for the production or consumption of various compounds were calculated from the slopes of the data points using linear regression.

2.4. *Analytical methods*

All filtered samples were injected into vials (with or without preservative) and stored on ice till the entire batch test was completed. Samples were then taken directly to the analytical laboratory for immediate analysis. The time between sampling and chemical analysis varied between 0.5 and 8 h. Dissolved sulfur species (SO_4^{2-}, HS^-, $S_2O_3^-$ and SO_3^{2-}) were analyzed using the IC (Dionex ICS-2000) and the method described in Gutierrez et al. (2008). Dissolved nitrogen species (NO_3^- and NO_2^-) were analyzed on the IC in vials without SAOB. VFA, soluble and total COD were measured using methods described in Gutierrez et al. (2008). Methane was analyzed using a gas chromatogram (Shimadzu GC-9A equipped with a flow injection detector) using the protocol described in Guisasola et al. (2008). Dissolved oxygen was measured with a YSI InPro 6050 oxygen sensor connected to a 4100 Metller Toledo O_2 transmitter, and pH was analyzed using a pH probe with a TPM-miniCHEM process monitor and controller.

2.5. *Microsensor measurements of RM1 biofilm*

Microsensor experiments on RM1 biofilm of the experimental line were carried out to provide information about microbial biofilm activity at a micrometer scale. Biofilm carriers were removed from the reactor and mounted in a flow cell (as detailed in Gutierrez et al., 2008) containing 140 mL of 0.22 μm filtered wastewater and 20 mL of 300 mM phosphate buffer (added to ensure a stable pH of 7.0–7.5). Nitrogen gas (99.99% purity) was bubbled through the measurement chamber to ensure anaerobic conditions and to provide mixing. Microsensors were mounted on a micromanipulator and positioned on the surface of the biofilm using a dissection microscope. The concentration gradients through the biofilm were recorded by moving the microsensors in increments of 25–100 μm and recording the sensor signal at each depth. The thickness of the biofilm was roughly estimated using an eyepiece micrometer and the depth of penetration adjusted accordingly. Three to five replicate profiles were obtained per experiment. A static diffusive boundary layer approximately 200 μm thick was consistently present between the biofilm and the liquid phase in all profiles.

Microsensors for oxygen, nitrous oxide and nitrate were constructed and calibrated before every experiment according to Revsbech (1989), Andersen et al. (2001) and De Beer et al. (1997), respectively. Hydrogen sulfide sensors (Kuhl et al., 1998) and pH (Revsbech and Jørgensen, 1986) sensors were purchased from Unisense A/S (Denmark), and calibrated according to procedures described in Mohanakrishnan et al,

(2009) and Revsbech and Jørgensen (1986), respectively. The tip diameters of all sensors were between 5 and 15 μm.

Steady-state profiles were obtained by incubating the biofilm for 1 h in the medium before measurements were made. Oxygen profiles were measured to confirm anaerobic conditions. Total sulfide profiles were calculated from the sulfide and pH profiles obtained as described in Kuhl et al. (1998). From these profiles, the diffusive sulfide fluxes from the biofilm surface to the bulk liquid were calculated from the concentration gradient through the diffusive boundary layer above the biofilm using Fick's first law. The sulfide production zones within the biofilm were calculated using the diffusion reaction model developed by Berg et al. (1998). The diffusion coefficient used for total sulfide in the biofilm was 1.39×10^{-5} cm^2 s^{-1} at 20 °C (Kuhl and Jørgensen, 1992), based on the assumption that the diffusion coefficients within the biofilm were equal to the molecular diffusion coefficients. This value is taken from the literature and therefore has associated uncertainties. Measurement of the diffusion coefficient in the experimental biofilm using a diffusion sensor was not recommended due to the thinness of the biofilm in comparison to the typical tip diameter of such sensors (>100 μm). The porosity was assumed to be close to unity and constant with depth in the biofilm. These assumptions may influence the calculated sulfide production rates; however, since they are consistent between all experiments, they are not expected to influence the interpretation of results. Bulk substrate conditions were monitored by analyzing liquid samples drawn over the course of the experiment for dissolved sulfur and VFA using methods described in Section 2.4. Table 2 summarizes the objectives and conditions of the microsensor experiments.

Table 2 – Summary of RM1 biofilm microsensor tests.

Objective of studies	No. of studies	Wastewater conditions	Sensors used
To measure the long-term effects of nitrate on the sulfide production capabilities of the biofilm	2 (phase 1); 6 (phase 2)	Fresh wastewater	Sulfide, pH
To investigate biofilm adaptation to nitrate on a spatial scale within the biofilm	1 (phase1); 2 (phase 2: day 0–10 h, and day 17)	Fresh wastewater + nitrate added (20 mg-N L^{-1})	Sulfide, pH
To investigate the effects of different bulk nitrate concentrations on sulfide production	1 (phase 2)	Fresh wastewater + nitrate added in separate experiments (0 mg-N L^{-1}; 2 mg-N L^{-1}; 4 mg-N L^{-1}; 8.8 mg-N L^{-1}; 11.5 mg-N L^{-1}; 16.3 mg-N L^{-1} and 24 mg-N L^{-1})	Sulfide, pH, nitrate, nitrous oxide
To investigate the activity of nitrate-driven biological sulfide oxidation in the biofilm	1 (phase 2)	Wastewater with high sulfate (14.4 mg-S L^{-1}) and low VFA (<4 mg-COD L^{-1}) + nitrate added (10.2 mg-N L^{-1}) + sulfide added (15.0 mg-S L^{-1})	Sulfide, pH, nitrate

2.6. *Microbial structure and composition of RM1 biofilm*

2.6.1. *Denaturing gradient gel electrophoresis (DGGE)*

Microbial community fingerprints were obtained on day −27 (phase 1), and days 4 and 32 (phase 2) from RM1 biofilm of the experimental line using DGGE. DNA extraction and polymerase chain reaction (PCR) of the 16S rRNA and *dsr* genes were performed as described in Mohanakrishnan et al, (2009) and Gutierrez et al. (2008), respectively. Positive (*Desulfotomaculum* clone) and negative (no template) controls were included for all reactions, and sizes of the PCR products were verified on 1.5% agarose gels. DGGE was carried out on an 8% acrylamide gel with a 30–70% denaturing gradient. Electrophoresis and staining procedures were conducted as described in Mohanakrishnan et al, (2009). Bands consistently present in the biofilm from the different phases were excised and sequenced (Macrogen, Inc). Six partial sequences of the 16S rRNA gene and eight partial sequences of the *dsr* gene were submitted to GenBank (accession nos. EU426849–EU42685 and EU426855–EU426862 respectively), and similarity analysis was done using the BLASTN Search program (http://www.ncbi.nlm.nih.gov/BLAST).

2.6.2. *Physical characteristics*

Biofilm carriers were also removed from RM1 of the experimental line on selected days in phase 1 and phase 2 and analyzed for solids using the protocol described in Gutierrez

et al. (2008). The changes in colour and visual appearance of the biofilm under the microscope as a result of nitrate addition were also monitored.

3. Results

3.1. Long-term impact of nitrate addition on wastewater sulfur transformations and methane discharge

The wastewater composition at the outlets of the experimental and reference lines during pseudo steady-state of phase 2 is presented in Table 3. Sulfide concentrations at the outlet of the reference line fluctuated between 7 and 15 mg-S L^{-1} (average inlet sulfate concentration was 15 mg-S L^{-1}), with more sulfide generated in periods with infrequent pumping, i.e. with longer wastewater HRT (Fig. 2).

Nitrate addition decreased wastewater sulfide discharges from the experimental line by 66% in comparison with the reference line. However, total sulfide control was not achieved in the experimental line, as seen from sulfide discharges at the outlet (Fig. 2). High levels of sulfide were measured in the experimental line during low flow periods with infrequent pump cycles (between 12 am and 6 am and between 2 pm and 5 pm), when nitrate in the bulk had depleted. No sulfide was discharged during high flow periods (between 7 am and 12 pm and between 7 pm and 11 pm) when nitrate was not completely consumed due to the shorter HRT in these periods.

Nitrate addition raised the pH of the wastewater being discharged to 7.9–8.3, a consequence of denitrification (Table 3). This is potentially beneficial to a sewer system as hydrogen sulfide emission to the gas phase will be reduced when a rising main receiving nitrate discharges wastewater into a gravity sewer. As might be expected, nitrate addition incurred carbon consumption in the experimental line and approximately 200 mg-COD L^{-1} (40% of the inlet COD) was consumed for 30 mg-N L^{-1} of nitrate. Assuming this nitrate was exclusively used for heterotrophic denitrification, the COD:N ratio is in general agreement with the denitrification reaction stoichiometry. Interestingly, a loss of 140 mg-COD L^{-1} (30% of inlet COD) was also observed in the reference line (Table 3). Sulfate reduction to sulfide (Fig. 2) contributed to this loss of COD. According to the reaction $CH_3COOH + H_2SO_4 \rightarrow 2CO_2 + 2H_2O + H_2S$, 33.4 mg-COD L^{-1} should have been consumed for the reduction of 11.7 mg-S L^{-1} sulfate to sulfide. Biomass growth, which was ignored in the above calculation, would have further increased COD consumption. The remaining COD loss was attributed to methane production. As seen in Fig. 3, methane concentrations of 70–100 mg-COD L^{-1} were discharged from the reference line during an 8 h period (daytime). The discharge concentrations again correlated with the frequency of pump events. In contrast to the reference line, no methane discharge was observed in the experimental line (Fig. 3), and the measured values at the outlet were similar to that observed at the inlet (~5 mg-COD L^{-1}) of this line. These results indicate that nitrate addition effectively suppressed methane discharge from the experimental line.

3.2. Comparison of SRB activity in the experimental line during phases 1 and 2

The maximum SRB sulfide production rates in the four reactors of the experimental line are presented in Fig. 4. Nitrate addition did not have any long-term inhibitory/toxic effect on the sulfide production capability of the biofilm in RM1-3 (p-test: null hypothesis, no significant difference, $p > 0.05$). In fact, it caused an increase in sulfide production in RM4 ($p < 0.05$). Measurement of diffusive sulfide fluxes out of RM1 biofilm in the experimental line using microsensors revealed a similar lack of change in SRB capability as a result of nitrate addition (Fig. i, Supplementary material). These results explain why sulfide accumulated in the wastewater upon depletion of nitrate (Fig. 2). The 32% increase in the RM4 sulfide production capability was likely caused by SRB enrichment in RM4, due to increased sulfate availability in this section of the sewer as a consequence of little net sulfate consumption in the upstream sections when nitrate was present.

3.3. Adaptation of RM1 biofilm to nitrate addition

Further insights on the impact of nitrate on SRB activity in sewer biofilm were gained at the beginning of phase 2, when

Table 3 – Performance parameters of the reference and experimental lines with standard errors ($n = 5$).

Parameters	Reference line	Experimental line
Amount of NO_3 dosed (mg-N day^{-1})	0	360
Amount of sulfide discharged (mg-S day^{-1})	140.5 ± 1.5	47.6 ± 2.6
pH	7.2-7.5	7.9-8.3
Average sulfide in the outlet/pump event (mg-S L^{-1})	11.7 ± 0.3	4.0 ± 0.2
Sulfide control effectiveness (%)	-	65.9 ± 2.0
Inlet VFA concentration (mg-COD L^{-1})	67.8 ± 0.6	67.8 ± 0.6
Outlet VFA concentration (mg-COD L^{-1})	2.13 ± 0.5	1.07 ± 0
Inlet SCOD concentration (mg-COD L^{-1})	265 ± 0.5	265 ± 0.5
Outlet SCOD concentration (mg-COD L^{-1})	194.2 ± 12.5	156.6 ± 15.9
Carbon consumption (% of S-COD consumed)	27	41
Inlet TCOD concentration (mg-COD L^{-1})	469 ± 0.6	469 ± 0.6
Outlet TCOD concentration (mg-COD L^{-1})	327.2 ± 15.6	269 ± 17.9
Carbon consumption (% of T-COD consumed)	30	43

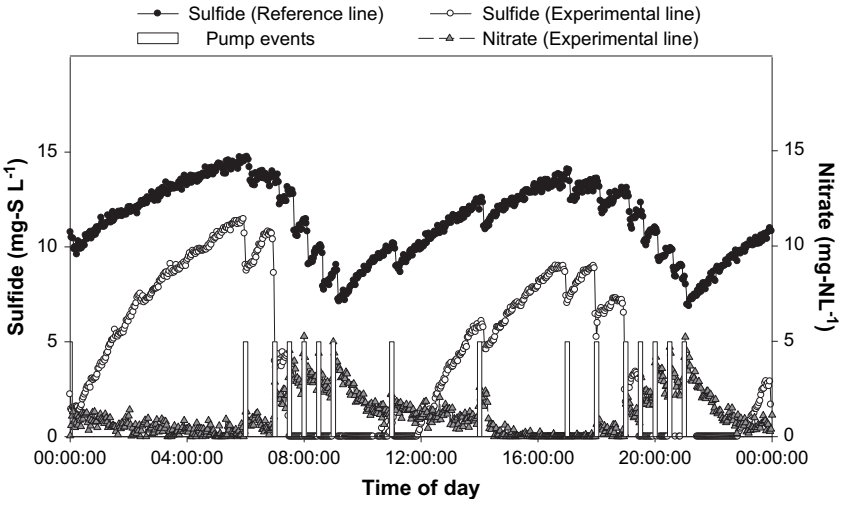

Fig. 2 – Twenty-four hour profile of sulfide at the outlet of the reference line, and of sulfide and nitrate at the outlet of the experimental line. The vertical columns indicate pump events. The distinct pattern of sulfide curves generated was a result of sulfide production in quiescent periods, and dilution of wastewater slugs during pumping.

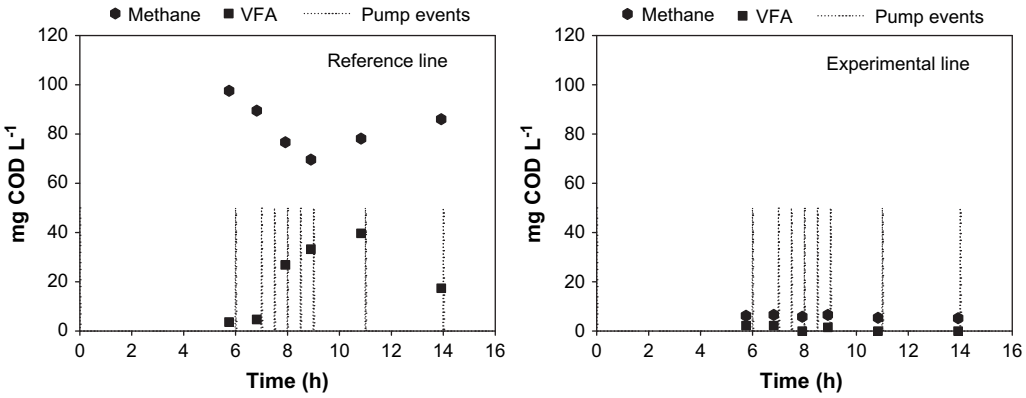

Fig. 3 – Methane and VFA concentrations at the outlet of the reference and experimental lines measured just before each pumping event. The dotted lines in the graphs represent pumping events.

the biofilm was adapting to nitrate exposure. Table 4 summarizes the rates of sulfate reduction, sulfide production and nitrate reduction in RM1 during the first few nitrate exposure events, and Fig. 5 shows the detailed sulfur and nitrogen wastewater transformations in two of these tests. Sulfate reduction and sulfide production continued in the presence of nitrate in RM1 for the first three nitrate exposure events, with rates comparable to or even higher than that measured prior to nitrate addition (background). The mere presence of nitrate therefore did not stop sulfate reduction in the biofilm. However, no net sulfate reduction and sulfide production was observed in RM1 in the presence of nitrate by four nitrate exposure events (Fig. 5b and Table 4) and with subsequent exposures (data not shown). This rapid change in behaviour, discussed further in Section 3.4, was attributed to the development of anoxic sulfide oxidation within the biofilm. The nitrate consumption rate increased steadily with repeated exposure to nitrate (Table 4). This rate averaged

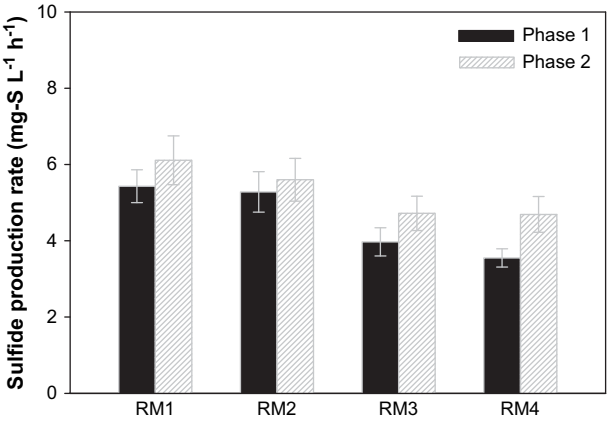

Fig. 4 – Mean sulfide production capabilities of different sections of the experimental line with standard errors, under non-limiting substrate conditions in the absence of nitrate. $n = 4$; 13 in phases 1 and 2, respectively.

Table 4 – Adaptation of RM1 to nitrate.

No. of nitrate doses	Sulfate reduction rate (mg-S L^{-1} h^{-1})	Sulfide production rate (mg-S L^{-1} h^{-1})	Nitrate reduction rate (mg-N L^{-1} h^{-1})
0	3.80	3.97	0
1	4.80	4.64	0.83
2	7.33	6.22	1.57
3	6.28	5.43	2.31
4	0	0	3.52

33 mg-N L^{-1} h^{-1} during the pseudo steady state of phase 2 which was substantially higher than that measured in the first few exposure events (Fig. ii, Supplementary material). No nitrous oxide production was detected within the sewer biofilm from nitrate reduction (data not shown).

The microscale sulfide profiles in RM1 biofilm during and after adaptation to nitrate are presented in Fig. 6. Oxygen profiles confirm the negligible presence of oxygen within the biofilm during these tests. When 20 mg-N L^{-1} nitrate was added for the first time, little change was seen in the sulfide production throughout RM1 biofilm (Fig. 6a). However, in biofilm previously exposed to nitrate for 10 h, there was less sulfide accumulation in the presence of nitrate, compared with that in the absence of nitrate (Fig. 6b). The negative slope at depths of 400–700 μm in this profile indicates there was consumption of sulfide in the biofilm below the depths analyzed. This consumption is likely linked with sulfide oxidation by nitrate (discussed further in Section 3.4). No net sulfide production was detected in the presence of nitrate in nitrate-adapted biofilm (profile measured on day 17 of phase 2) (Fig. 6c), and sulfide consumption was observed throughout the biofilm. The diffusive fluxes of sulfide from Fig. 6 experiments are presented in Table i (Supplementary material).

3.4. *Biological versus chemical sulfide oxidation with nitrate*

A rapid decrease of sulfide was observed, concurrently with nitrate reduction, when nitrate was added to wastewater containing sulfide and lacking VFA, providing evidence of combined biological and chemical sulfide oxidation with nitrate (Fig. 7a). The sulfur balance reveals that only part of the sulfide was oxidized to sulfate. The loss in total dissolved sulfur (sum of measured sulfate, sulfide, sulfite and thiosulfate) suggests the formation of intermediate products from partial sulfide oxidation with nitrate. This loss was not observed in phase 1 or in the reference line (data not shown), suggesting that these compounds were exclusively formed in the presence of nitrate and retained in the biofilm. These compounds were not measurable using the methods applied, and require further investigation. Yang et al. (2005) have previously suggested the formation of elemental sulfur during sulfide oxidation with nitrate. The sulfate reduction, sulfide production, nitrate reduction and nitrite accumulation rates measured in five similar experiments are summarized in Table 5.

Similar anoxic experiments were also conducted in a reactor without biofilm (Fig. 7b, Table 5). Chemical sulfide oxidation with nitrate was insignificant when compared to biological sulfide oxidation. The sulfur balance shows that sulfate was the primary product of chemical sulfide oxidation with nitrate. This is supported by the ratio between nitrate consumption (1.7 mg-N L^{-1} h^{-1} or 0.12 mmol-N L^{-1} h^{-1}) and sulfate production (1.9 mg-S L^{-1} h^{-1} or 0.06 mmol-S L^{-1} h^{-1}), which approximately matches the stoichiometry of the chemical reaction, $H_2S + 1.6\ HNO_3 \rightarrow H_2SO_4 + 0.8N_2 + 0.8H_2O$, and findings of Yang et al. (2004).

The difference in sulfide oxidation rates between the batch tests (Table 5) suggests that the removal of sulfide in the presence of nitrate is primarily microbial. Biological sulfide oxidation was further demonstrated within the biofilm (Fig. iii, Supplementary material) using microsensors. Maximum activity was seen in the upper 200 μm of the biofilm.

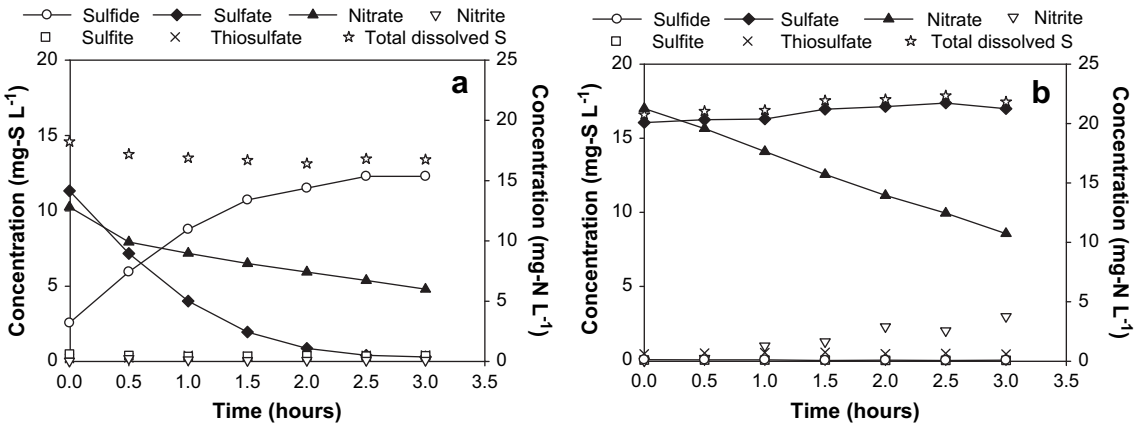

Fig. 5 – Changes in dissolved sulfur and nitrogen species in RM1 during (a) the second and (b) fourth nitrate exposure events.

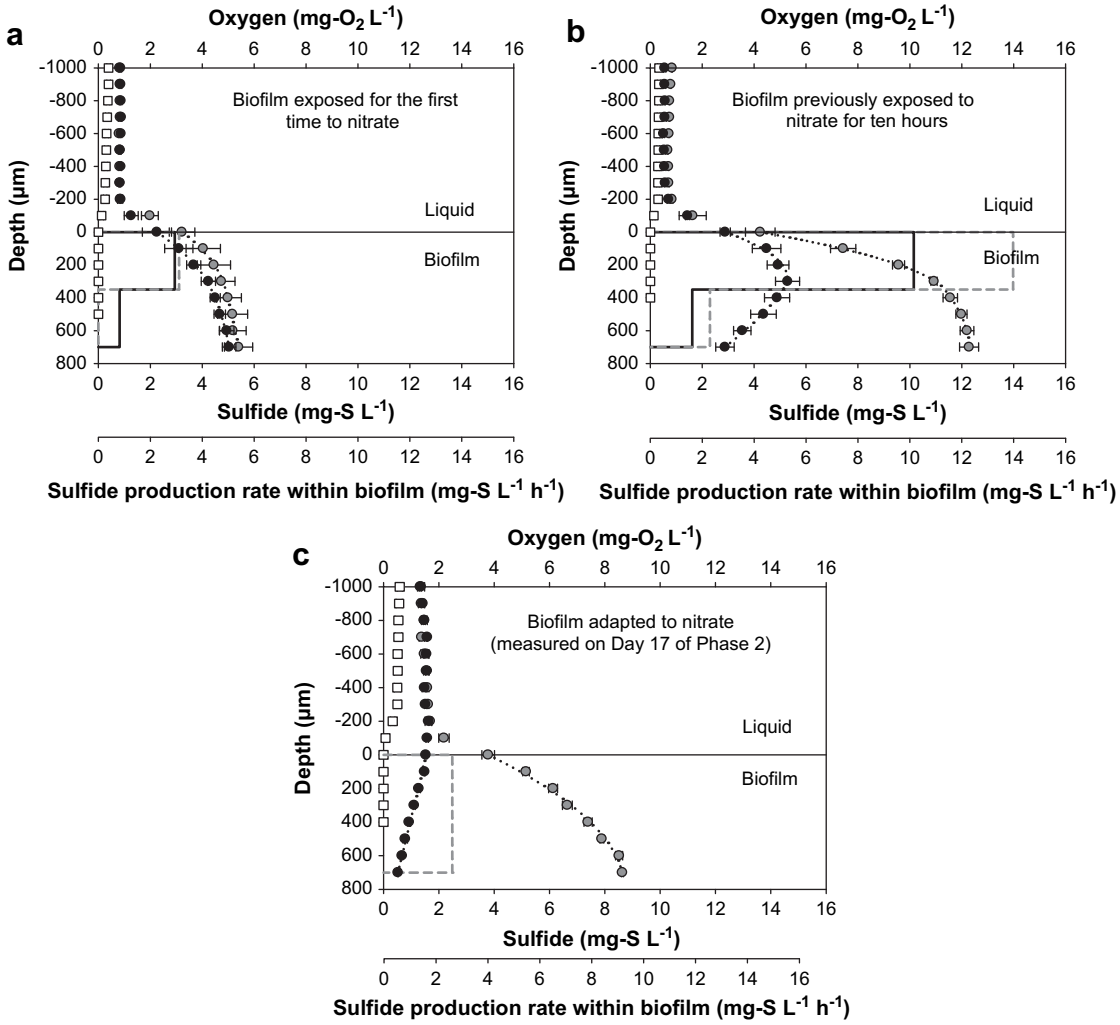

Fig. 6 – Total sulfide (mg-S L^{-1}) profiles with standard errors ($n = 3$) in the absence (●) and presence (●) of nitrate (20 mg-N L^{-1}). The biofilm surface was defined as depth 0 µm. Production profiles of sulfide in the absence and presence of nitrate are plotted using broken lines and full lines, respectively. Dotted lines are the modelled sulfide concentration gradients within the biofilm corresponding exactly to the production profiles. Oxygen (□) concentrations were zero within the biofilm.

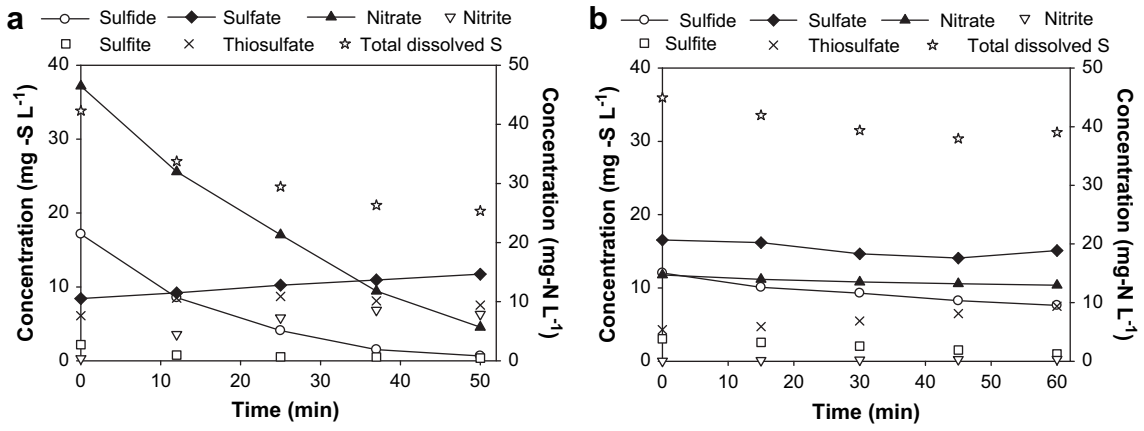

Fig. 7 – Typical batch tests measuring (a) combined biological and chemical sulfide oxidation with nitrate, and (b) chemical sulfide oxidation with nitrate.

Table 5 – A comparison of the rates observed with combined biological and chemical sulfide oxidation (with standard errors, $n = 5$), and with chemical sulfide oxidation with nitrate.

Batch tests (mean ± SE)	Sulfide oxidation (mg-S L^{-1} h^{-1})	Sulfate production (mg-S L^{-1} h^{-1})	Nitrate consumption (mg-N L^{-1} h^{-1})	Nitrite production (mg-N L^{-1} h^{-1})
Combined biological and chemical sulfide oxidation	54.8 ± 6.9	6.7 ± 0.9	76.5 ± 9.3	18.2 ± 5.7
Chemical sulfide oxidation	4.3	1.9	1.7	0.3

In spite of effective biological sulfide oxidation with nitrate, the lack of SRB inhibition implies that certain nitrate concentrations are required in the bulk to achieve a sulfide oxidation rate exceeding that of sulfate reduction. Fig. 8 shows the diffusive flux of sulfide out of nitrate-adapted RM1 biofilm at various nitrate concentrations. The individual profiles are presented in Fig. iii (Supplementary material). A linear decrease ($R^2 = 0.93$) at a rate of 3.65 mg m^{-2} h^{-1}/(mg-N L^{-1}) was observed in the sulfide diffusive flux out of the biofilm on increasing nitrate concentrations in the bulk from 0 to 11.5 mg-N L^{-1}. At higher nitrate concentrations, no sulfide diffused out of the biofilm for the duration that nitrate was present. This implies that a minimum nitrate concentration of approximately 12 mg-N L^{-1} was required to completely suppress any sulfide accumulation in the bulk. However, this threshold value was determined in the presence of non-limiting carbon substrates. Deeper penetration of nitrate into the biofilm is expected when the COD concentration in the bulk is less, which would result in a lower threshold value.

3.5. *Biofilm community composition and physical properties*

As might be expected from the lack of SRB activity inhibition, SRB populations in the biofilm were not affected by nitrate addition (Fig. v, Supplementary material), and a similar population diversity was observed on days −27, 4 and 32 with *dsr*-DGGE. Selected bands (G-N) were sequenced and revealed >80% sequence similarity to an uncultured SRB clone (GenBank no. AM181120), *Desulfobulbus rhabdoformis* (GenBank accession no. AJ250473), *Syntrophobacter* fumarooxidans (GenBank accession no. AF418193), *Desulfobulbus elongatus* (GenBank accession no. AJ310430), *Desulfomicrobium apsheronum* (GenBank accession no. AF482459), an uncultured SRB isolate (GenBank accession no. EF065047), an uncultured *Desulfotomaculum* clone (GenBank accession no. DQ415718) and *Desulfovibrio vulgaris* (GenBank accession no. CP000527) respectively. A gene-based PCR approach was preferred over the other bacterial identification techniques like fluorescence *in situ* hybridization due to lack of initial knowledge of the dominant phylogenetic SRB subgroups in rising main sewer biofilm, and the inherent potential to track changes in these bacterial populations as a result of nitrate addition.

No significant changes were also observed in general microbial diversity, obtained by DGGE of 16S rRNA gene products (Fig. v, Supplementary material). Some bands changed in intensity but the total number of bands present in the 16S rRNA-based DGGE lanes remained constant. Bands A–F, common to all the samples were sequenced and showed maximum similarity (>95%) to a number of heterotrophic Gram-positive bacteria and Gram-negative *Proteobacteria* including an uncultured *Bacteroides* clone (GenBank accession no. EF111174), *Azonexus caeni* (GenBank accession no. AB166882), *Acidovorax* sp. (GenBank accession no. Y18616), an uncultured bacterium related to *Comamonas* sp. (GenBank accession no. AB286581), *Aquabacterium* sp. (GenBank accession no.. AF089856) and *Desulfovibrio carbinolicus* (GenBank accession no. DQ186201) respectively. *Desulfovibrio* was the only genus of SRB detected using 16S rRNA-based DGGE, indicating that members of this genus were abundant, even after the addition of nitrate.

The visual appearance of the RM1 biofilm changed from dark green-brown in phase 1 to a mix of dark green and lighter whitish-brown patches by the end of phase 2 (image not shown). The thickness of the RM1 biofilm remained almost unchanged during phase 1 and phase 2 (Fig. vi, Supplementary material).

4. Discussion

4.1. *Nitrate does not inhibit SRB activity in sewer biofilm*

Continuous addition of nitrate to RM1 did not affect the capability of RM1-4 biofilm to produce sulfide (Fig. 4) and nitrate addition to sewers is therefore not expected to have a long-

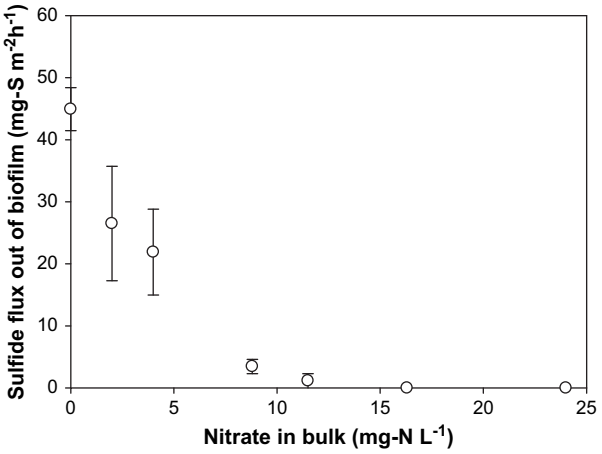

Fig. 8 – Mean sulfide diffusive flux from the biofilm at increasing nitrate concentrations, with standard errors. $n = 5$.

lasting inhibitory/toxic effect on the sulfide production capability of sewer biofilm (in the absence of nitrate). Fig. 5a further shows that sulfate reduction and sulfide production continued in the wastewater even in the presence of nitrate during the first exposure event. This suggests that nitrate addition does not have an instantaneous inhibitory effect on sulfate reduction in anaerobic sewer biofilm. There are two possible explanations for this phenomenon. The first one is that SRB activity continues unhindered despite exposure to nitrate. The alternative is that SRB activity is not inhibited because some of the bacteria are protected from exposure to nitrate due to mass transfer limitations under quiescent condition and nitrate consumption by other heterotrophic bacteria in the biofilm, or due to positioning in anaerobic microniches where their activity can continue unhindered. Okabe et al. (2003) have previously demonstrated sulfate reduction in narrow anaerobic zones in microaerophilic wastewater biofilm exposed to nitrate. These zones had the potential to migrate deeper within the biofilm or to expand upward depending on the bulk conditions. However, as seen in Fig. 6a, the sulfide production throughout RM1 biofilm in the presence of 20 mg-N L^{-1} nitrate was almost identical to that measured in the absence of nitrate. This supports the first hypothesis, namely that SRB activity is not inhibited despite exposure to nitrate. The lack of complete nitrate penetration into the biofilm at low bulk nitrate concentrations permits sulfide production in the lower anaerobic layers (Fig. iii, Supplementary material), and explains the immediate resumption of sulfide accumulation after nitrate depletion (Fig. 2).

Microbial community fingerprinting confirmed there was no significant change in the SRB populations in RM1 biofilm after the addition of nitrate, suggesting that the SRB populations detected in phase 1 survived well in phase 2 and were likely responsible for the sulfate reduction capabilities of RM1 biofilm (Fig. 4). Some of the SRB populations detected in RM1 biofilm, in particular *Desulfovibrio* and *Desulfobulbus*, have been identified in laboratory-grown gravity sewer biofilm (Ito et al., 2002). Substrate uptake patterns of these groups, identified using a combination of microautoradiography and fluorescence *in situ* hybridization, revealed that *Desulfobulbus* was the most active and dominant subgroup for the uptake of both [^{3}H]- and [^{14}C]propionate, with nitrate as an electron acceptor (Ito et al., 2002). Similar lack of major SRB population changes with nitrate treatment has also been observed in laboratory-grown biofilm from oil production water (Kjellerup et al., 2005), suggesting a generic lack of impact of nitrate on SRB microbial diversity.

4.2. *Biological sulfide oxidation with nitrate plays an important role in sulfide control in nitrate-receiving sewers*

No apparent sulfate reduction was observed in RM1 biofilm after four nitrate exposure events (Fig. 5b). This is hypothesized to be a consequence of simultaneous sulfide oxidation as it is being produced, which results in no accumulation of sulfide in the bulk. Supporting this hypothesis is Fig. 6b, where it is seen that biofilm previously exposed to nitrate for 10 h has lower sulfide accumulation in the presence of nitrate. The negative slope in the deeper biofilm layers indicates sulfide oxidation in the layers below. No net sulfide production was detected in the nitrate-adapted biofilm (Fig. 6c), and sulfide consumption was observed throughout the biofilm. The biological sulfide oxidation capability of the biofilm clearly increases with repeated nitrate exposure events, and explains the lag time observed in Saracevic et al. (2006). This adaptation is necessary for nitrate addition to effectively prevent sulfide diffusion from the biofilm.

Biological sulfide oxidation with nitrate was confirmed by comparing the oxidation in the presence and absence of biomass (Fig. 7). Nitrate reducing, sulfide oxidizing processes have been demonstrated in recent years (De Gusseme et al., 2009; Garcia-de-Lomas et al., 2007). It is likely that as the sewer biofilm adapted to nitrate exposure, the activity of autotrophic denitrifiers, the NR-SOB, increased and eventually exceeded sulfide production (by SRB), leading to zero net sulfide production in the biofilm in the presence of nitrate (Figs. 6c, 5b and Table 4). Given the rapid onset of such activities (hours), these organisms were possibly already present in the sewer biofilm, in line with the findings by Garcia-de-Lomas et al. (2007) under different environmental conditions, and required a relatively short period of time for enzyme activation and for growth. Gevertz et al. (2000) reported average NR-SOB doubling times of 1.3–2.9 h, which fits well with the short biofilm adaptation period observed.

Maximum biological sulfide oxidation activity (Fig. iii, Supplementary material) was seen in the upper 200 μm of the biofilm. This suggests a higher NR-SOB abundance in these upper biofilm layers. Such a spatial positioning would allow NR-SOB to procure nitrate from the bulk, and sulfide from SRB activity within the biofilm. Increased activity in these layers would in turn, effectively limit sulfide diffusion from the biofilm in the presence of nitrate. The nitrate concentration in the bulk will also determine the rate of sulfide diffusion from the biofilm (Fig. 8).

Another factor that could have contributed to the lack of sulfide accumulation in nitrate-adapted biofilm in the presence of nitrate is the possibility for SRB to have switched their activity from sulfate reduction to nitrate reduction (more energetically favourable) with repeated nitrate exposure events, resulting in reduced sulfide production rates. Such a switch has been previously demonstrated in pure culture studies (Mitchell et al., 1986). Further, SRB from the *Desulfovibrio*, *Desulfobulbus* and *Desulfomonas* genera have been shown to effectively reduce nitrate and gain energy for growth (Ito et al., 2002; Krekeler and Cypionka, 1995). They do so by inducing the nitrate reductase and/or nitrite reductase enzymes, responsible for the conversion of nitrate to nitrite and for the conversion of nitrite to ammonia, respectively (Moura et al., 1997). We identified bacteria with high similarity to these genera (*Desulfovibrio*, *Desulfotomaculum*) in sewer biofilm; however, identification and quantification of this switching activity require further investigations.

4.3. *Nitrate suppresses methane discharge in sewers*

Methane production has previously been detected in rising main sewers and it is now understood that greenhouse gas emissions as a consequence of gas release during sewer discharge contributes significantly to greenhouse gas emissions from wastewater systems (Guisasola et al., 2009). Methane production in sewers therefore ideally needs to be controlled. This study shows that addition of nitrate for

sulfide control has a favourable side effect on methane control (Fig. 3). Methane suppression under anoxic conditions has been studied in mixed methanogenic communities and is attributed to the formation of denitrification intermediates like nitric oxide and nitrous oxide (Kluber and Conrad, 1998; Tugtas and Pavlostathis, 2007). The level of inhibition observed in these assays depended on the concentration of the nitrogen compound and the type of bacterium present. Decreased methanogen activity may also have been a result of altered redox conditions in the presence of nitrate or due to chemical oxidation of methane by nitrate. Methanogenesis resumed in the experimental line some hours after nitrate depletion (data not shown), suggesting that the inhibition effect of nitrate was reversible. However, a detailed investigation is required before further conclusions can be made.

4.4. *Improving the effectiveness of nitrate addition on sulfide control in sewers*

Nitrate addition to sewers had no inhibitory effects on SRB activity and did not alter the major SRB populations in sewer biofilm. This has significant implications for the use of nitrate for sulfide management, as it reveals that the complete reduction of sulfide discharges is possible only if the wastewater is maintained under anoxic conditions for the entirety of its travel. As such, both the nitrate dosing location and concentration are important. Nitrate should be added at a point close to the end of the sewer (or close to the point of sulfide control), yet giving an adequate wastewater retention time from the point of nitrate addition to the point of sulfide control to allow for complete oxidation of sulfide formed upstream. The dosage rate should be such that the entire section between the two points is maintained anoxic. Given the dynamic hydraulic flow patterns in a sewer system, the dosage rate should ideally be controlled dynamically to meet the varying nitrate demand at different times over the course of a day. The need for dynamic dosage is clearly seen from Fig. 2, which showed that constant nitrate dosage resulted in nitrate depletion in some periods, and that nitrate was discharged (and thus wasted) in other periods. Another option would be to use multiple dosing locations. A station closer to the point of control can be used during periods with low wastewater flow. The minimized nitrate addition will not only reduce the chemical consumption operational costs, but may also be beneficial for the downstream wastewater treatment plants performing biological nutrient removal due to reduced consumption of biodegradable COD in wastewater (Table 3).

Nitrate addition upstream increases the sulfide production capability of downstream biofilm (Fig. 4), and as a result, increases the overall capability of the sewer for sulfide production in the absence of nitrate. Consequently, a portion of the sulfide removed during the high flow period could in fact be returned during the low flow period. This further highlights the importance of maintaining anoxic conditions in locations downstream of the dosage point. It must also be remembered that sulfide control with nitrate addition requires an adaptation period to stimulate the biological sulfide oxidation within the biofilm. Another factor to be considered in designing the dosage of flow is that the nitrate uptake capability of the biofilm increases with repeated exposure to nitrate (Fig. ii, Supplementary), and a dosage station should not be sized based on the nitrate consumption rates measured in the first few days of trial. Further studies are required to demonstrate the practical and financial feasibility of nitrate optimization.

5. Conclusions

This study demonstrates that nitrate addition has substantial impacts on the wastewater characteristics and the activities of sewer biofilm. The main conclusions made are:

- Nitrate does not inhibit SRB activity in the short- or long-term, and does not decrease the abundance of SRB in the sewer biofilm. In fact, nitrate addition to the start of rising mains increase SRB activity in downstream biofilm.
- Nitrate addition is overall effective in controlling sulfide concentrations in rising main sewers. The effectiveness of nitrate relies on its sustained presence in the bulk.
- Nitrate addition stimulates biological sulfide oxidation within the sewer biofilm after a short period of adaptation. This adaptation period is necessary for the effectiveness of nitrate on sulfide control.
- Nitrate drastically reduces methane discharge in rising mains.

The incorporation of these findings into nitrate dosing strategies will allow the optimized use of nitrate for sulfide control in rising main sewers.

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Supplementry data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.watres.2009.06.021.

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