RESEARCH ARTICLE

Effect of nitrate on sulfur transformations in sulfidogenic sludge of a marine aquaculture biofilter

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Abstract

The effect of NO_3^- addition on dissimilatory SO_4^{2-} reduction and sulfide conversion in organic-rich sludge from the digestion basin of a recirculating marine aquaculture system was studied. SO_4^{2-} reduction could only explain a minor fraction (up to 4–9%) of the observed total sulfide production (up to 35 mmol L^{-1} day^{-1}), indicating that the main source of sulfide in the sludge was not SO_4^{2-} reduction, but desulfuration during the decomposition of organic matter. Although NO_3^- inhibited SO_4^{2-} reduction, but not desulfuration, the primary NO_3^- mitigation effect was the onset of NO_3^--mediated sulfide oxidation (up to 75 mmol L^{-1} day^{-1}), partially to elemental sulfur (S^0). Above NO_3^- concentrations of 0.6 mM in the bulk water, the net sulfide production and oxidation zones were moved deeper into flocs and sludge cores, which effectively prevented sulfide from entering the water column. However, the sulfide efflux from the sludge instantly recovered after NO_3^- depletion. Thus, the NO_3^- level in the water column controls the zonation and magnitude of sulfur transformations in the sludge. The effect of NO_3^- relies therefore on its sustained presence in the water column, which in turn depends on a well-functioning nitrification in the mariculture system.

Introduction

Zero-discharge marine aquaculture (mariculture) systems provide a solution for the environmentally detrimental effects of conventional aquaculture in the sea (Cytryn *et al.*, 2003, 2005a). These recirculating systems comprise biofilters for the aerobic and anaerobic treatment of waste products from the fish tank, i.e. NH_4^+ and particulate organic matter. The drawback of an anaerobic treatment stage is the potential for sulfide production, especially in marine systems, in which high levels of SO_4^{2-} contained in the seawater provide the basis for SO_4^{2-} reduction (Cytryn *et al.*, 2003). Because sulfide causes massive fish mortality already at micromolar concentrations (Bagarinao, 1992), the control of sulfide production and its efflux into the recirculating water is of crucial importance to sustain the functional stability of closed mariculture systems.

Sulfide production has been mitigated by the addition of NO_3^- in various engineered and natural systems, such as sewers (Heukelekian, 1943), wastewaters (Vigneron *et al.*, 2007), oil reservoirs (Thorstenson *et al.*, 2002), and eutrophic freshwater wetlands (Lucassen *et al.*, 2004). However, the underlying mechanisms and the long-term impact of the nitrogen input on these habitats are debated. NO_3^- addition may control SO_4^{2-} reduction by (1) exclusion of SO_4^{2-}-reducing prokaryotes (SRP) by NO_3^--reducing bacteria (NRB) through competition for available electron donors (Hubert & Voordouw, 2007); (2) inhibition of the dissimilatory sulfite reductase, the key enzyme of SRP, by NO_2^- produced during NO_3^- reduction in NRB (Greene *et al.*, 2006); and (3) a metabolic switch of certain SRP, which prefer NO_3^- over SO_4^{2-} as terminal electron acceptor (Seitz & Cypionka, 1986; and references therein). In addition, NO_3^--dependent sulfide oxidation can remove substantial amounts of sulfide and thereby counteract the net sulfide production of the anaerobic treatment stage. In fact, the presence and activity of NO_3^--reducing sulfide-oxidizing bacteria (NR-SOB) have been implicated previously in a recirculating mariculture system by 16S rRNA gene analysis and chemical profiling (Cytryn *et al.*, 2003).

The present study focused on the anaerobic, organic-rich sludge of the digestion basin (DB) of a recirculating mariculture system with the goals to (1) identify the contribution of SO_4^{2-} reduction to overall sulfide production, and (2) to

determine the effect of NO_3^- on SO_4^{2-} reduction and sulfide efflux. Microsensor measurements in single sludge flocs and intact sludge cores as well as ^{35}S-SO_4^{2-} bulk incubations were utilized to assess the stratification and magnitude of SO_4^{2-} reduction/sulfide production with and without NO_3^- added.

Materials and methods

System description

The mariculture system (Supporting Information, Fig. S1) was launched as a pilot plant in 1998 in Rehovot, Israel (Cytryn *et al.*, 2003), and consisted of a fish tank connected to a nitrifying trickling filter, an anaerobic DB for the removal of particulate organic matter (feces and waste feed), and a fluidized bed reactor to ensure complete sulfide removal. The fish tank was stocked at a high density with gilt-head sea bream (*Sparus aurata*) in artificial seawater (ASW); fish were fed with a commercial mixture containing 45% protein and 19% fat (Matmor Ltd, Evtach, Israel). Initial microsensor and molecular analyses were performed with sludge from the DB of the pilot plant in fall 2003. In 2004, a full-scale system (Fig. S1) was established; the sludge from the pilot DB was transferred to the full-scale DB, and the pilot plant was closed down. All further experiments were performed with sludge from the full-scale plant in spring 2005; both systems had been continuously operated for >6 months before the measurements.

The DB of the pilot plant has been described in detail previously (Cytryn *et al.*, 2003); the full-scale DB (area, 3.6 m^2; working volume, 1.5–3.0 m^3; flow rate, 0.8 $m^3\,h^{-1}$; hydraulic retention time, 5 h; Fig. S1) was filled with 0.4–0.6 m^3 sludge, mainly consisting of fish feces (2–3 kg dry weight (d.w.) day^{-1}), uneaten feed (<0.4 kg day^{-1}), and bacterial biomass. The sludge bed was 11–18 cm thick, and was covered by 40 cm of water. The total organic matter content and density of the sludge were 46–48% d.w. and 0.05–0.07 g d.w. cm^{-3}, respectively. The water had a salinity of 20 ± 2‰, a SO_4^{2-} concentration of 14 mM, an average pH of 7.5, and a temperature of 20–27 °C.

Initial analysis of the pilot plant sludge

Sludge was sampled at three positions in the pilot plant DB (close to the inlet, middle, and outlet) and immediately frozen for molecular analyses. DNA was extracted as described previously (Foesel *et al.*, 2008); the gene coding for dissimilatory sulfite reductase (*dsrAB*) in SRP was amplified, and PCR products were pooled, cloned, and sequenced according to published protocols (Kjeldsen *et al.*, 2007). Single flocs from the same sludge were attached with a drop of agar (40 °C) to the bottom of a flow cell ($100 \times 50 \times 50$ mm; made of plexiglas) for microsensor measurements. Filtered ASW (salinity, 20‰; pH 8.0) prepared from Red Sea Salt (Red Sea Fish Pharm Ltd, Eilat, Israel) and amended with either lactate, acetate, or acetate plus $NaNO_3$ (1 mM each) was circulated at a flow velocity of 1 cm s^{-1} through the flow cell from a 5-L aquarium (Stief & Eller, 2006). The O_2 concentration was maintained ≤ 3 μM at the floc surface by purging the water

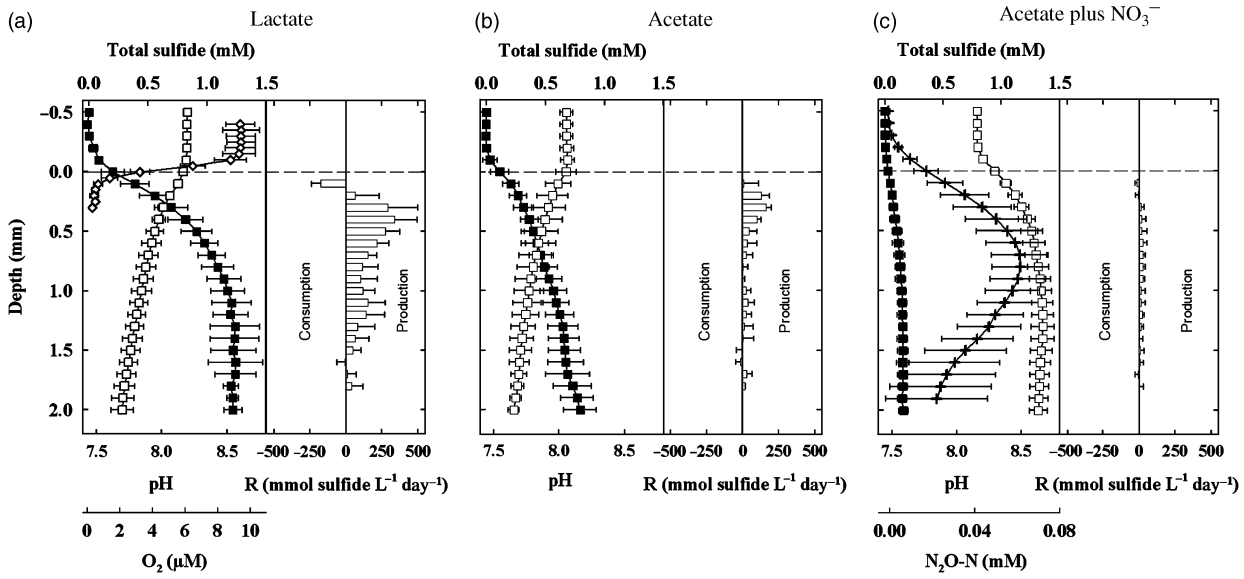

Fig. 1. Concentration profiles of total sulfide (bold rectangles), pH (open rectangles), and N_2O (crosses) in single sludge flocs from the pilot plant DB in the presence of lactate (a), acetate (b), and acetate plus NO_3^- (c). Volume-specific net sulfide conversion rates inside the flocs are shown on the right. The O_2 concentration inside all measured flocs was typically below 3 μM ($n=9$ profiles) as shown in a representative O_2 profile (a; diamonds). Zero depth equals the floc surface. Error bars indicate the SD of at least four measurements in different flocs.

with N_2 (Fig. 1a). The flocs were allowed to adapt to the experimental conditions for at least 2 h before microprofiling of O_2, H_2S, pH, and N_2O with glass microsensors that were prepared and calibrated as described previously (Revsbech & Jørgensen, 1986; Revsbech, 1989; Jeroschewski *et al.*, 1996; Andersen *et al.*, 2001). Microsensors were controlled by a motorized micromanipulator and profiling was performed using the software M-PROFILER (L. Polerecky, MPI Bremen). The total sulfide profiles were determined from the local H_2S concentration that was corrected for the prevailing pH and temperature using a pK_1 of 7.02 (Millero *et al.*, 1988). N_2O profiling was combined with the acetylene block method (Sørensen, 1978) to determine denitrification rates from the accumulation of N_2O in NO_3^--amended flocs exposed to 10% acetylene. For each parameter, four to seven replicate profiles were measured in two to four flocs.

Bulk experiments with full-scale sludge

Sludge was sampled at four positions in the full-scale DB (see Fig. S1b), i.e. from the *top* 1–2 mm and from the *bottom* of the sludge bed, at both the *inlet* and the *outlet* of the DB. Samples were collected into Erlenmeyer flasks using a 100-mL glass tube (Sher *et al.*, 2008) and flushed immediately with N_2; subsamples (6 mL) were directly frozen for later chemical analysis. To determine the gross SO_4^{2-} reduction rates (SRR) at ambient NO_3^- concentrations at the four DB positions, 2-mL sludge was transferred in replicates into 12-mL gas-tight glass vials (Exetainer, Labco, UK) for radiotracer incubations (see below). To determine the effect of NO_3^- addition on SRR, equal volumes of sludge from all four DB positions were pooled, stirred, and preincubated under a flow of N_2 for 1.5 h to remove internal NO_3^- via denitrification. Subsequently, 2 mL of the pooled sludge was transferred in replicates into gas-tight glass vials for radiotracer incubations, and aliquots of sterile $NaNO_3$ in ASW were added to final concentrations of 0, 0.1, 0.8, 4, and 6 mM NO_3^-, respectively.

SRR analysis in bulk sludge

The headspace of the incubation vials was flushed with helium gas after sealing. All vials received 10 μL of carrier-free radiolabeled ^{35}S-SO_4^{2-} tracer (Amersham Bioscience) to a final total activity of 50 kBq per vial. The total SO_4^{2-} concentration in the samples ranged between 1.3 and 9.9 mM in the sludge from the four DB positions (Table 1) and 3.0 and 4.7 mM in the pooled sludge. Duplicate or triplicate vials were incubated horizontally on a rotator in the dark at 23–25 °C. The incubations were stopped after a maximum of 10 h by adding 5 mL of zinc acetate (20% w/v) to the sludge. Negative controls were prepared by adding 5 mL of zinc acetate (20% w/v) to the sludge before radiotracer addition. Gross SRR determinations were performed using the cold chromium distillation procedure as described in Kallmeyer *et al.* (2004), and the radioactivity was determined by liquid scintillation counting (Packard 2500 TR). The liquid phase of the sludge was obtained from additional sludge samples that had not received radioactivity and were incubated in parallel to the tracer-amended vials. Liquid-phase samples were immediately frozen at −20 °C until the analysis of NO_3^-, NO_2^-, and sulfide (sum of H_2S, HS^-, and S^{2-}). Gross SRR were calculated from the generation of ^{35}S-SO_4^{2-} in the presence of NO_3^- and after NO_3^- depletion.

Microprofiling in sludge cores

The effect of NO_3^- in the water column on the zonation of sulfur conversions in the sludge was measured using microsensors. Undisturbed cores were collected using a Plexiglas cylinder (5 cm diameter, 7.5 cm height) from the sludge bed of the full-scale DB downstream the *inlet* position (Fig. S1b). Cores were placed in a 6-L aquarium with recirculating ASW, which was continuously flushed with argon and mixed at a velocity of 1 cm s^{-1} using a submersible pump. NO_3^- concentrations were adjusted by the addition of $NaNO_3$ to 0.1, 0.6, 0.9, and 4.5 mM. Floating balls (Allplas, Capricorn

Table 1. Conversion rates and concentrations in sludge from the full-scale DB at ambient NO_3^- concentrations

DB position	Gross SO_4^{2-} reduction rate (mmol L^{-1} day^{-1}) ($n=2$)	% Sulfide released via SO_4^{2-} reduction	Net sulfide production rate (mmol L^{-1} day^{-1})	NO_3^- (mM) ($n=2$)	NO_2^- (mM)	SO_4^{2-} (mM) ($n=2$)	Total S (mol L^{-1}) ($n=4$)	Total N (mol L^{-1}) ($n=4$)	Total C (mol L^{-1}) ($n=4$)
Inlet									
Top	0.40	4.0	10	2.2	0.003	5.5	0.12 ± 0.02	2 ± 0	13 ± 1
Bottom	0.21	0.6	35	0.0	0.005	1.3	0.09 ± 0.00	2 ± 0	14 ± 1
Outlet									
Top	0	NA	0	3.1	0.004	9.9	0.13 ± 0.00	2 ± 0	13 ± 2
Bottom	0.15	0.5	31	0.9	0.003	1.5	0.11 ± 0.04	1 ± 0	13 ± 0

Gross SO_4^{2-} reduction rates were obtained from ^{35}S-SO_4^{2-} trace incubations, and the net sulfide production rates were calculated from liquid-phase sulfide concentrations in bulk sludge samples (Pachmayr, 1960; Kallmeyer *et al.*, 2004). The NO_3^-, NO_2^- and SO_4^{2-} values stated here are the concentrations in the liquid phase of the sludge; bulk water concentrations of NO_3^- and SO_4^{2-} were 3.3 and 14 mM, respectively.
NA, not applicable.

Chemicals Corp., Secaucus, NY) covered the water surface to minimize gas exchange with the atmosphere. Bulk water O_2, pH, and temperature were continuously monitored with macrosensors (WTW, Weilheim, Germany). Replicate profiles of pH, H_2S, and O_2 were recorded at several positions and replicate cores as described above. A maximum solubility of 21.19 mmol L^{-1} for N_2O was used (Weiss & Price, 1980).

Rate calculations

The total conversion rates of sulfide, NO_3^-, and O_2 were obtained via stepwise calculation of the local conversion rates (Gieseke & de Beer, 2004), which were deduced from concentration profiles, and by integrating local conversion rates over the entire depth of activity inside flocs (1.8 mm) and sludge cores (20 mm), respectively. A planar geometry was assumed for all calculations. The diffusion coefficients (D_0) in water for H_2S, N_2O, and NO_x were 1.69×10^{-5}, 2.28×10^{-5}, and 1.84×10^{-5} $cm^2\,s^{-1}$ (Broecker & Peng, 1974; Li & Gregory, 1974); the effective diffusion coefficient in the sludge (D_{eff}) was taken as $0.95 \times D_0$ (Christensen & Characklis, 1990).

Chemical analyses

Sulfide was determined spectrophotometrically at 663 nm using the methylene blue method of Pachmayr (1960) as detailed by Trueper & Schlegel (1964). NO_3^- and NO_2^- were quantified on a NO_x chemiluminescence detector (Thermo Environmental Instruments, Franklin, MA) as described previously (Braman & Hendrix, 1989). The total C, N, and S contents of the sludge were measured on an elemental analyzer (NA 1500 Series 2; Fisons Instruments). SO_4^{2-} was quantified from the liquid phase of the sludge by routine nonsuppressed anion chromatography using a Waters 510 pump (flow, 1.0 mL min^{-1}), a Waters IC-Pak anion exchange column, and a Waters 430 conductivity detector; the eluent was isophthalic acid (1 mM).

Results

Initial analysis of the pilot plant sludge

Analysis of 27 *dsrAB* clones revealed 14 OTUs (cutoff, 97% amino acid similarity) and the Good's coverage of the *dsrAB* library was 79%; sequences are available from GenBank under accession numbers EU350964–EU350990. The library only contained *dsrAB* sequences affiliated with deltaproteobacterial sulfate-reducing bacteria (SRB) and was dominated by sequences indicative of the genera *Desulfotignum* and *Desulfovibrio* (see Fig. S2 for details).

When single sludge flocs were incubated with either lactate or acetate, the O_2 concentration decreased from around 3 μM at the floc surface to 0 within the first 0.05 mm of the floc (Fig. 1a). In the absence of NO_3^-, sulfide was produced below this oxic–anoxic interface, presumably by SO_4^{2-} reduction during the oxidation of lactate or acetate rather than through sulfur mineralization, as internal organic matter providing desulfuration became depleted during the preincubation of the flocs. Concomitant to sulfide production was the decrease in pH from pH 8.1 in the overlying water to 7.7 toward the floc center (Fig. 1a and b). The net sulfide production within the first 1.8 mm was three times higher (128 ± 25 mmol L^{-1} day^{-1}) with lactate as electron donor than with acetate (42 ± 13 mmol L^{-1} day^{-1}). Sulfide was consumed in the oxic layer above the production zone at the water–sludge interface; this consumption was however incomplete so that some sulfide diffused into the water (Fig. 1a and b).

The addition of NO_3^- plus 10% acetylene to the acetate-incubated flocs resulted in a N_2O peak, indicative of denitrification, between 0.2 and 1.3 mm depth inside the flocs, and in a gradual increase of the pH with depth by > 0.5 U (Fig. 1c). The mean net volumetric denitrification rate determined from the N_2O profile was 15 ± 6 mmol N L^{-1} day^{-1}. The net sulfide production was reduced to 33% of the production rate without NO_3^- (14 ± 9 mmol L^{-1} day^{-1}); sulfide was completely consumed already in the sludge and did not enter the water phase (Fig. 1c). Upon increasing the NO_3^- concentration in the water column to 6 mM, white precipitates were observed on the surface of the flocs (see Fig. S3a).

Sulfur conversions in the full-scale DB

Both SO_4^- and NO_3^- concentrations were lower in the liquid phase of the sludge than in the overlying water and decreased from the *inlet* to the *outlet* and from *top* to *bottom* sites, with NO_3^- fully depleted at the *inlet bottom* site (Table 1); these gradients imply the reduction of both SO_4^{2-} and NO_3^- in the sludge. NO_2^- was only present at low concentrations throughout the DB (average, 3 μM), and the total C, N, and S concentrations did not differ significantly between the sampling points (Table 1).

Net sulfide production was the highest in the *bottom* sludge samples, where NO_3^- concentrations were low or depleted; no net sulfide production occurred in the *outlet top* sludge, where NO_3^- concentrations were the highest. Gross SRR ranged from 0.15 to 0.40 mmol L^{-1} day^{-1}, with the highest rate in the *inlet top* sludge and no detectable SO_4^{2-} reduction in the *outlet top* sludge; only a maximum of 4% of the total sulfide produced originated from SO_4^{2-} reduction (Table 1).

Effect of NO_3^- addition on bulk sulfur conversions

Experimental addition of NO_3^- to bulk sludge pooled from the full-scale DB resulted in decreasing SRR (Fig. 2, Table 2). SRR correlated negatively with the initial NO_3^-

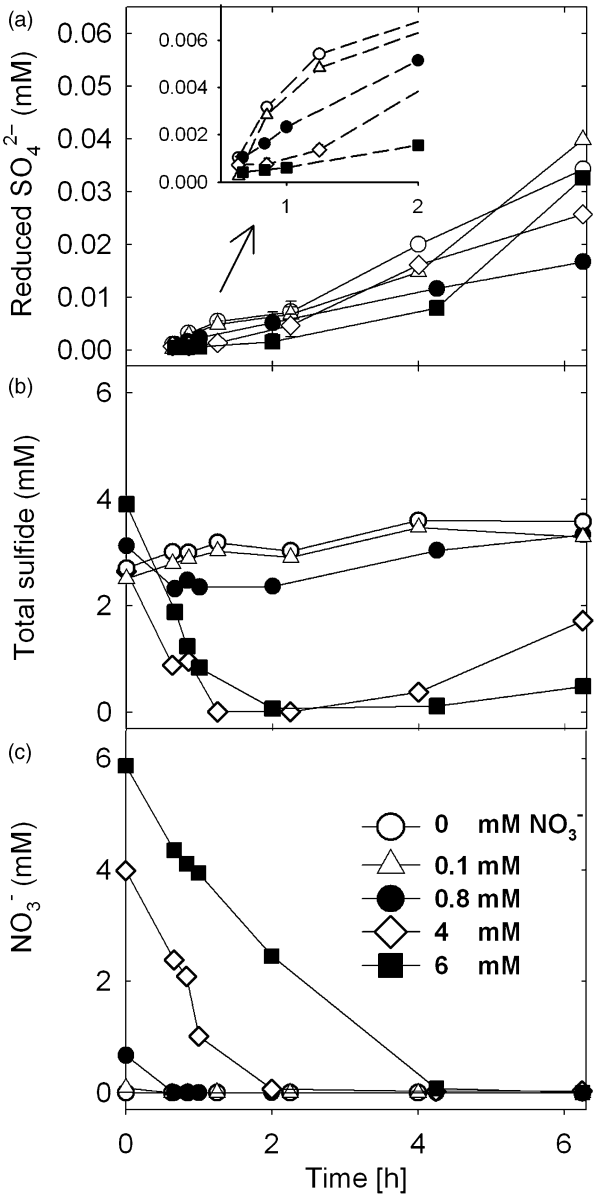

Fig. 2. Time course of total (labeled and unlabeled) reduced SO_4^{2-} (a), sulfide (b), and NO_3^- (c) concentrations at different initial NO_3^- levels (0, 0.1, 0.8, 4, and 6 mM) in pooled sludge from the full-scale DB. Average values from duplicate measurements are shown (error bars were omitted). For clarity, the total reduced SO_4^{2-} concentrations from the first 2 h are enlarged in (a).

concentrations (Fig. 3), and SRR were reduced by up to 94% at the highest NO_3^- concentration tested (6 mM; Fig. 3). Likewise, the net sulfide production rates decreased, and at a NO_3^- concentration of 0.8 mM, became negative, indicating that sulfide was actually oxidized. Sulfide oxidation was mirrored by NO_3^- reduction, with a stoichiometric (1 : 1) relation of sulfide oxidation and NO_3^- reduction rates (NRR), and both positively correlated with the initial NO_3^- concentration in the liquid phase of the sludge (Table 2). The fraction of sulfide originating from SO_4^{2-} reduction was below 2% (Table 2).

After NO_3^- in the liquid phase of the sludge was depleted, both SRR and net sulfide production increased immediately (Fig. 2) and reached similar values as in the treatment without NO_3^- addition; up to 9% of the total sulfide now originated from SO_4^{2-} reduction (Table 2).

Effect of NO_3^- addition on the stratification of sulfur conversions

Microsensor measurements in intact sludge cores showed an oxic surface layer of approximately 2 mm depth; the O_2 penetration depth was unaffected by NO_3^- in the overlying water (Fig. 4a). In the absence of NO_3^- (data not shown) and at the lowest NO_3^- concentration tested (0.1 mM), sulfide production was detected directly below the oxic–anoxic interface (Fig. 4a and b), and sulfide accumulated to up to 9 mM at 30 mm depth (data not shown). The volume-specific net sulfide production rate within the first 20 mm of the sludge, as derived from microprofiling, was 1.1 ± 0.9 mmol L^{-1} day^{-1}; sulfide oxidation in the oxic surface layer was 0.13 mmol L^{-1} day^{-1}, and sulfide occasionally diffused into the water phase. The pH declined from 8.5 at the water–sludge interface to 7.6 at 20 mm depth (Fig. 4a).

With increasing NO_3^- concentrations in the water phase, sulfide oxidation progressed deeper into the anoxic zone, and net sulfide production was detected deeper in the sludge (Fig. 4a and b). NO_3^- concentrations above 0.6 mM effectively suppressed the efflux of sulfide to the overlying water; addition of 4.5 mM NO_3^- resulted in a negative volume-specific net sulfide production rate within the first 20 mm of the sludge bed (-2.2 ± 0.6 mmol L^{-1} day^{-1}), i.e. in the net removal of sulfide. The anaerobic sulfide oxidation zone was characterized by an increased pH (8.4–8.6) compared with the sludge surface and the sulfidogenic zone below (Fig. 4a). During the first hour following NO_3^- addition, white precipitates, presumably elemental sulfur from incomplete sulfide oxidation, accumulated on the sludge surface (Fig. S3b).

Discussion

Sources of sulfide

SO_4^{2-} reduction is the dominant carbon mineralization process in most marine and coastal sediments (Jørgensen, 1982; Skyring, 1987), where it represents the main source of biogenic sulfide (Widdel, 1988). In analogy, SO_4^{2-} reduction is assumed (Cytryn *et al.*, 2005a, 2006; Neori *et al.*, 2007) to be the main source of sulfide in mariculture systems, which therefore seem to be more difficult to operate than

Table 2. Average conversion rates at different experimental NO_3^- levels in pooled sludge from the full-scale DB as determined from Fig. 2

	Initial rates (with NO_3^- present)				Rates after NO_3^- depletion		
Initial [NO_3^-] in the liquid phase of the sludge (mM)	Gross SO_4^{2-} reduction rate (mmol L^{-1} day^{-1}) ($n = 2$)	% Sulfide released via SO_4^{2-} reduction	Net sulfide production rate (mmol L^{-1} day^{-1})	NO_3^- reduction rate (mmol L^{-1} day^{-1})	Gross SO_4^{2-} reduction rate (mmol L^{-1} day^{-1}) ($n = 2$)	% Sulfide released via SO_4^{2-} reduction	Net sulfide production rate (mmol L^{-1} day^{-1})
0	0.17	1.9	9	NA	0.15	4.9	3
0.1	0.17	1.7	10	3	0.20	9.3	2
0.8	0.09	NA	−29	28	0.12	6.7	2
4	0.04	NA	−49	56	0.11	1.1	10
6	0.01	NA	−75	48	0.26	4.7	6

NA, not applicable.

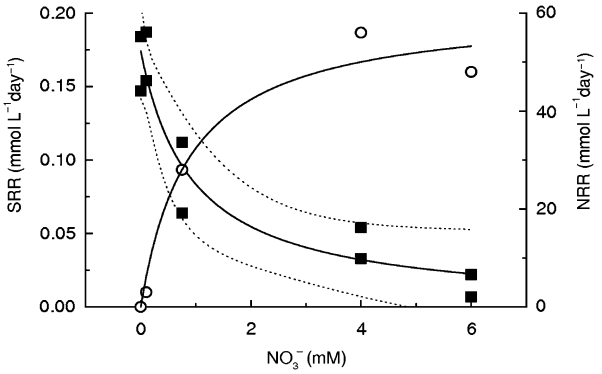

Fig. 3. Effect of NO_3^- on the initial gross SRR (closed squares) and NRR (open circles) in pooled sludge from the full-scale DB. The rates were determined from the curves in Fig. 2. Dotted lines represent the 95% confidence limits of the fitted curve for SRR ($n = 2$).

SO_4^{2-}-poor freshwater aquacultures. SO_4^{2-} reduction in the DB sludge was indicated by the detection of functional marker genes (*dsrAB*) of SRB (Fig. S2) and by counter gradients of SO_4^{2-} and sulfide in the sludge bed (Table 1, Fig. 4), and was confirmed by direct measurements in single sludge flocs and bulk sludge (Tables 1 and 2; Fig. 1). The threefold increased net sulfide production with lactate compared with acetate in single flocs (Fig. 1) may be partially explained by the 50% higher reduction potential during SO_4^{2-} reduction of lactate compared with acetate and partially by the community composition of the SRB (Rabus *et al.*, 2006): with acetate alone, only the complete oxidizing SRB in the sludge (mostly *Desulfotignum*-related SRB, Fig. S2) will be active, while lactate would also stimulate the incomplete oxidizers (mostly *Desulfovibrio*-like SRP; see Figs S2 and S1; Cytryn *et al.*, 2003).

Gross SRR in the bulk sludge (0.15–0.40 mmol L^{-1} day^{-1}) were comparable to the rates reported for organic-rich sediments, for example, below aquacultures (Holmer & Kristensen, 1992) or coastal up-welling regions (Bruechert *et al.*, 2003), as well as in eutrophic and SO_4^{2-}-enriched freshwater lakes (Holmer & Storkholm, 2001); still, SO_4^{2-} reduction could only explain a minor fraction (up to 4%) of the observed total sulfide production of up to 35 mmol L^{-1} day^{-1} (Tables 1 and 2).

These results imply that the main source of sulfide in the sludge was not SO_4^{2-} reduction, but desulfuration during organic matter decomposition. In fact, the sludge contained high amounts of S-rich particulate organic matter, reflected in the high total sulfur contents (Table 1), and the daily sulfur load of the DB by feeding (approximately 0.5 mol) can easily account for the observed sulfide production rates. Furthermore, typical bacteria involved in the decomposition of particulate organic matter (e.g. *Bacteriodetes*, *Dethiosulfovibrio*, *Clostridium*) have been reported previously in sludge from the pilot-scale DB (Cytryn *et al.*, 2003).

Effect of NO_3^- on SO_4^{2-} reduction

NO_3^- generally caused a concentration-dependent inhibition of SO_4^{2-} reduction (Fig. 3). Although drastically decreased, SO_4^{2-} reduction was not completely eliminated (Figs 1, 2a and 3) and resumed immediately after NO_3^- depletion (Fig. 2b; Table 2). The most likely mechanism for this inhibition is the ability of NRB to outcompete SRB for limited electron donors, for example, volatile fatty acids (Hubert & Voordouw, 2007). Alternative explanations appear to be less probable: firstly, NO_2^-, which may be produced via NO_3^- reduction and can inhibit the dissimilatory sulfite reductase in SRP (Greene *et al.*, 2006), never exceeded μM concentrations in the system (Table 1). Secondly, the metabolic switch from SO_4^{2-} reduction to NO_3^- reduction has not been described for any of the SRB identified in the DB (Kuever *et al.*, 2001; Rabus *et al.*, 2006). If the competition hypothesis is correct, the extent of the inhibition will depend on both the NO_3^- and the electron donor concentration (Hubert *et al.*, 2003). This may explain why SRR, under ambient NO_3^- levels in the DB (Table 1), were the highest at the *inlet top* site despite a NO_3^- concentration of 2.2 mM; close to the inlet, most of the fresh organic carbon is oxidized (Cytryn *et al.*, 2003), and consequently volatile fatty acid

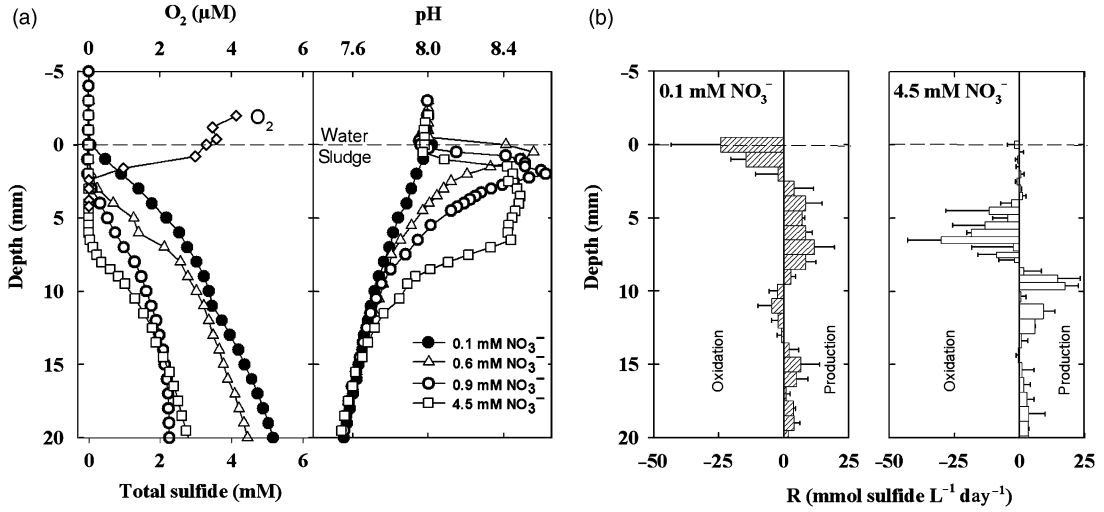

Fig. 4. Concentration profiles (a) of total sulfide, O_2 (diamonds) and pH in sludge cores from the full-scale DB at different NO_3^- levels in the overlying water. Average profiles of at least two measurements in cores from different positions in the DB are shown; error bars were omitted for clarity. Volume-specific net sulfide conversion rates (b) at 0.1 (hatched bars) and 4.5 mM (open bars) NO_3^- in the bulk water based on the corresponding microprofiles in (a). Zero depth indicates the sludge–water interface.

concentrations tend to be higher than in the rest of the DB (Foesel, 2007). In contrast, at the *outlet top* site, volatile fatty acids are essentially depleted (Foesel, 2007), and therefore SO_4^{2-} reduction is completely absent already at a NO_3^- concentration of 3.1 mM (Table 1).

Effect of NO_3^- on net sulfide production

Although NO_3^- addition inhibited SO_4^{2-} reduction, but not desulfuration, the main NO_3^- mitigation effect was the onset of NO_3^--mediated sulfide oxidation. Above NO_3^- concentrations of 0.6 mM in the bulk water, the net sulfide production and oxidation zones were moved deeper into flocs and sludge cores, which effectively prevented sulfide from entering the water column (Figs 1 and 4). Aerobic sulfide oxidation was limited to a narrow zone below the water–sludge interface and was quantitatively negligible (Figs 1 and 4); similarly, sulfide oxidation and NRR above 0.8 mM NO_3^- (Table 2) also indicated that most of the sulfide was oxidized anaerobically with NO_3^-. Candidate NR-SOB have been identified previously in the DB as members of the *Rhodobacteraceae* and the genus *Dethiosulfovibrio* (as indicated by Cytryn *et al.*, 2003, 2005b). Increasing pH in the sulfide oxidation zone of flocs and bulk sludge indicated that NO_3^- addition resulted in the (at least partially) incomplete oxidation of sulfide to S^0 rather than in (pH neutral) SO_4^{2-} production (Sayama *et al.*, 2005; Kamp *et al.*, 2006). Obvious proof for incomplete sulfide oxidation was the appearance of white S^0 precipitates on top of the bulk sludge and on the floc surface upon NO_3^- addition (Fig. S2), a phenomenon also occasionally observed on the sludge surface of both the pilot- and the full-scale DBs (Cytryn *et al.*, 2006; Neori *et al.*, 2007).

Conclusions

With the minor importance of SO_4^{2-} reduction and desulfuration as the main source of sulfide, the risk of sulfide poisoning does not appear to be more severe in the investigated mariculture than in freshwater systems, which also show episodic incidences of mass mortality due to the release of sulfide from organic-rich mud (Krom *et al.*, 1985; Bagarinao, 1992; Holmer & Storkholm, 2001). Addition of NO_3^- is effective in preventing sulfide from entering the bulk water, mainly by NO_3^--mediated sulfide oxidation in the anoxic sludge layers, while the inhibition of SO_4^{2-} reduction was quantitatively less relevant. The effect of NO_3^- relies on its sustained presence in the water column, which in turn depends on a well-functioning nitrification in the mariculture system.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Scheme of the full-scale mariculture system in the constellation operating during the investigation in spring 2005 and magnification of the digester (top-down view).
Fig. S2. Phylogenetic affiliation of *dsrAB* genes retrieved from the pilot plant sludge in 2004.
Fig. S3. Formation of elemental sulfur on the water–sludge interface observed during microprofiling after adding NO_3^- to flocs and sludge cores.

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