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Effects of finfish aquaculture on biogeochemistry and bacterial communities associated with sulfur cycles in highly sulfidic sediments

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ABSTRACT: A combination of biogeochemical analyses and molecular microbiological analyses were conducted to assess the environmental impact of finfish aquaculture and to elucidate the major microbial assemblages responsible for the production and removal of reduced sulfur compounds in fish-farm sediments. The average concentrations of H_2S (123 µM) and NH_4^+ (1310 µM) and the dissimilatory sulfite reductase (*dsr*) gene copy number (1.9×10^9 copies cm^{-3}) in the sediments at the farm site were 15-, 1.5- and 2-fold higher, respectively, than those measured at the less-impacted reference site. Accordingly, the sulfate reduction rate (SRR) at the farm site (118 mmol m^{-2} d^{-1}) was 19-fold higher than that measured at the reference site (6.2 mmol m^{-2} d^{-1}). Analyses of *dsrA* and 16S rRNA gene sequences revealed that the *Syntrophobacteraceae* and *Desulfobulbaceae* groups are the major sulfate-reducing bacteria around the fish-farm sediment. Interestingly, despite the high SRR (12.2–19.6 mmol m^{-2} d^{-1}), the H_2S concentration was low (<8 µM) in the top 0–2 cm of the fish-farm sediments. In this sulfide-mismatched zone, sulfur-oxidizing bacteria associated with *Gamma-* and *Epsilonproteobacteria* were abundant. Especially at the 1–2 cm depth, bacteria related to *Sulfurovum* in the *Epsilonproteobacteria* showed the highest relative abundance, comprising 62% of the 16S rDNA sequences. The results strongly suggest that *Sulfurovum*-like bacteria play a significant ecological and biogeochemical role in oxidation and reduction of reduced sulfur compounds from the organic-rich, highly sulfidic fish-farm sediments.

KEY WORDS: Aquaculture impacts · Benthic metabolism · Sulphidic environment · Sulfate reduction · Sulfate-reducing bacteria · Sulfur-oxidizing bacteria · *Sulfurovum*

INTRODUCTION

The global production of aquaculture, including finfishes, crustaceans, mollusks and other aquatic animals, increased from 2.6 million t in 1970 to 66.6 million t in 2012 (FAO 2010, 2014). Accordingly, the contribution of aquaculture to total global fisheries production doubled from 22.2% in 1996 to 42.2% in 2012 (FAO 2010, 2014). Although aquaculture provides a stable, high-quality food source for humans, large-scale industrial aquaculture activities inevitably cause severe environmental concerns by releasing large quantities of organic materials, such as uneaten fish feed and fecal pellets, to the surrounding coastal areas (Gray et al. 2002, Brooks & Mahnken 2003, Holmer et al. 2005, Kutti et al. 2007).

In organic-rich coastal sediments, oxygen is depleted quickly within a few mm of the surface sediment, and organic carbon (C_{org}) oxidation is dominated by anaerobic microorganisms relying on different terminal electron-accepting processes, such as denitrification and reduction of Mn(IV), Fe(III) and sulfate (Canfield et al. 2005). Because of the abundance of sulfate in seawater, sulfate reduction is

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regarded as the predominant C_{org} oxidation pathway in coastal sediments, especially in organic-enriched fish-farm sediments (Holmer et al. 2005, Hyun et al. 2013). Major environmental concerns regarding the dominance of C_{org} oxidation resulting from sulfate reduction in fish-farm sediments include the accumulation of highly toxic, reactive hydrogen sulfide (H_2S), which affects the diversity and community structure of the benthic macrofaunal and microbial communities (Lee et al. 2004, Bissett et al. 2006, Heilskov et al. 2006, Kutti et al. 2007, Castine et al. 2009, Kawahara et al. 2009, Yoon et al. 2009, Hargrave 2010, Valdemarsen et al. 2015, Jung et al. 2016).

Because large-scale aquaculture has such a large effect on sediment biogeochemistry and coastal ecosystems via benthic–pelagic coupling (Holmer et al. 2005, Strain & Hargrave 2005, Lee et al. 2011, Hyun et al. 2013), it is important to develop a tool to assess the environmental conditions of fish-farm sediments (MOF 2016). Total organic carbon content and concentrations of acid volatile sulfur compounds in the sediments have been proposed as chemical indicators for monitoring the environmental changes caused by fish farms and for evaluating farming capacity (Yokoyama 2003, Cho et al. 2013, Jung et al. 2016). However, the environmental assessment of fish-farm sediments relying solely on sulfur compounds is not useful for identifying major biogeochemical processes because these compounds are quickly recycled within the sediments (Canfield et al. 2005, Hyun et al. 2013, NIFS 2013). Additionally, biological indicators have been proposed for monitoring the environmental changes resulting from aquaculture. For example, changes of benthic macrofauna assemblages are generally recommended as a proxy for monitoring the impacts of cage farming (Macleod et al. 2008, Yoon et al. 2009, Jung et al. 2016). However, investigation of the benthic community using video surveillance and the identification of macrofauna and meiofauna to the level of individual species is expensive and requires professional skill to make an accurate assessment of the impact (Castine et al. 2009).

Alternatively, because bacterial communities respond quickly to environmental changes (Bissett et al. 2006, Castine et al. 2009, Kawahara et al. 2009), quantitative and qualitative analyses of the spatial distribution and metabolic activities of major microbial indicators may provide crucial information on the major C_{org} oxidation pathways and subsequent biogeochemical conversion of specific compounds that would be veiled by geochemical analysis alone (Vezzulli et al. 2002, Jørgensen 2006, Kondo et al. 2012a,b). However, little is known about the microorganisms directly responsible for the biogeochemical sulfur cycles in fish-farm sediments, where rapid recycling of sulfur compounds occurs (Asami et al. 2005, Bissett et al. 2006, Kondo et al. 2012a,b). The main objectives of this study were (1) to assess the environmental impact of finfish aquaculture in the sediments and (2) to elucidate microbial assemblages closely related to the production and removal of reduced sulfur from the surface sediments of a fish farm.

MATERIALS AND METHODS

Study area

The study area was located in Gamak Bay, a semi-enclosed bay near Yeosu on the southern coast of the Korean Peninsula (Fig. 1), where the aquaculture industry is heavily developed. In the study area, 2 main fish species, rockfish *Sebastes schlegeli* and sea bream *Pagrus major*, are cultured for 1.5 to 2 yr before they are harvested after October. To improve the commercial value of the farmed fish before shipment, a large quantity of fish food is distributed just before harvest (NIFS 2007). Sediment samples were collected on 15 September 2010, when the sediments received a high organic load from uneaten feed and fecal pellets. Two sampling sites were chosen: one directly underneath the fish cages (hereafter the farm site), and another frontward of the water flow located ~50 m in front of the fish cages (hereafter the reference site) to reduce direct impact by uneaten feed or fecal pellets (Fig. 1). Water depth was approximately 4 and 8.5 m at the farm and reference sites, respectively. The bottom water temperature and salinity were 24°C and 30.1 psu, respectively, at both sites. Dissolved oxygen (DO) concentration in the bottom water was 6.16 mg l^{-1} at both sites (NIFS 2010). Surface sediments at both sites were composed mostly of silt- and clay-sized sediment (NIFS 2010). *Capitella capitata*, a macrofaunal indicator of highly sulfidic conditions, was observed in the surface sediments of the farm site (NIFS 2010).

Sampling and handling

Sediment samples were taken before feeding to avoid disturbing the sediment condition. Triplicate sub-samples were collected for geochemical analyses using polycarbonate cores (6.5 cm i.d., 25 cm long) by SCUBA divers to minimize sediment distur-

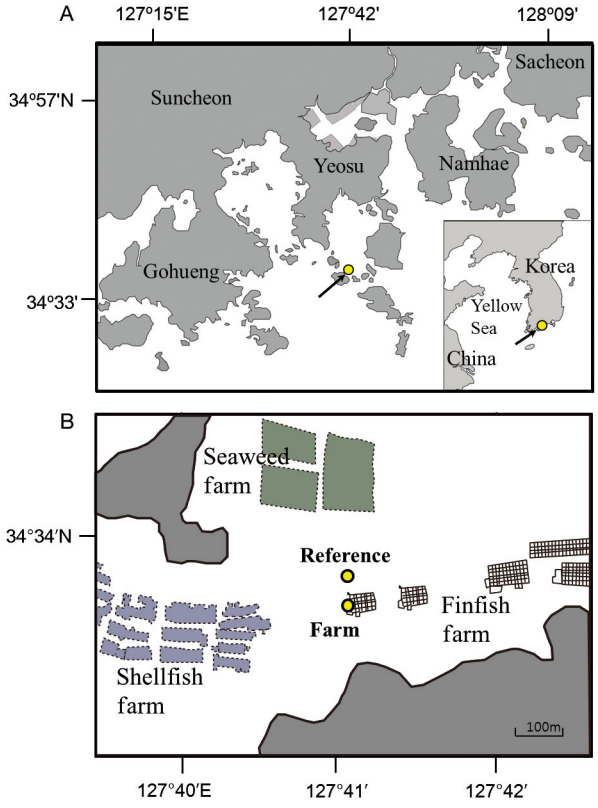

Fig. 1. (A) Study area in the coastal waters near Yeosu, South Korea; (B) '**farm**' indicates the sampling site where sediment samples were taken directly underneath the fish cages, whereas '**reference**' denotes the sampling site located ~50 m away from the fish cages

bance during sampling activities. In the N_2-filled plastic glove bag, the sediment core was sectioned into 1 cm intervals from the top of the core to a depth of 6 cm and at 2 cm intervals from 6 cm to 10 cm in depth. The sediments were then transferred to sterile centrifuge tubes (BD). The tubes were tightly capped and centrifuged for 10 min at 2700 × *g*. After reintroduction into the glove bag, porewater was sampled and filtered through 0.2 µm cellulose ester syringe filters (ADVANTEC). Duplicate samples of surface sediment (0–2 cm) for measuring particulate organic carbon (POC) and total nitrogen (TN) were frozen at –20°C until processed in the laboratory. Surface sediments for DNA extraction were collected using plastic cores that were pre-washed with sterilized water, rinsed with 70% ethanol and then stored at –80°C until analysis.

Porewater and solid-phase analyses

NH_4^+, NO_3^- and PO_4^{3-} concentrations in porewater were analyzed using an autoanalyzer (Proxima, Alliance; Hansen & Koroleff 1999). Dissolved sulfide was determined spectrophotometrically using the methylene blue method (Cline 1969) after precipitating sulfide in the filtered porewater with Zn acetate (20%). SO_4^{2-} concentration was measured in acidified porewater using ion chromatography (Metrohm 761). Dissolved Fe^{2+} was determined by the colorimetric method with Ferrozine (Stookey 1970). The detection limits of NH_4^+, NO_3^-, PO_4^{3-}, H_2S and Fe^{2+} were 0.021, 0.006, 0.007, 3 and 1 µM, respectively. The acetic acid concentration in porewater was analyzed using HPLC (Dionex Ultimate 2000).

Total oxalate-extractable Fe (hereafter total $Fe_{(oxal)}$) was extracted according to Thamdrup & Canfield (1996). Oxalate-extractable Fe(II) (hereafter $Fe(II)_{(oxal)}$) was extracted from frozen sediment in anoxic oxalate solution (Phillips & Lovley 1987). Both total $Fe_{(oxal)}$ and $Fe(II)_{(oxal)}$ were determined as described for the porewater analysis of Fe^{2+}. Oxalate-extractable Fe(III) (hereafter $Fe(III)_{(oxal)}$) was defined as the difference between the total $Fe_{(oxal)}$ and $Fe(II)_{(oxal)}$. POC and TN content were determined using a CHN analyzer (EA 1110; CE Instruments, Milan, Italy). The POC samples were treated with HCl (10%) to remove $CaCO_3$.

Rate of sulfate reduction

The microbial sulfate reduction rates (SRR) were determined in triplicate from intact cores (3 cm i.d., 35 cm long) according to the radiotracer (^{35}S-SO_4^{2-}, 15 kBq µl^{-1}, Institute of Isotopes) method of Jørgensen (1978). Five microliters of the ^{35}S-$SO4^{2-}$ working stock solution were injected into the sediment cores at 1 cm intervals. After 2 h of incubation at *in situ* temperature, the sediment was sliced into sections and then fixed in Zn acetate (20%) before being frozen immediately. Reduced ^{35}S extraction was performed using the 1-step distillation method (Fossing & Jørgensen 1989).

Nucleic acid extraction and quantification of the *dsr* gene

Total DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories) following the manufacturer's instructions. A quantitative PCR (qPCR) was used to determine copy numbers of the alpha subunit of the dissimilatory (bi)sulfite reductase gene (*dsrA*). The copy number of *dsrA* was used to quantify sulfate-reducing prokaryotes and was

determined using SYBR Green I assays, as described previously (Kondo et al. 2004, 2008, Liu et al. 2009). The primers DSR 1F (5′-ACS CAC TGG AAR CAC G-3′) (Wagner et al. 1998) and DSR R (5′-GTG GMR CCG TGC AKR TTG G-3′) (Kondo et al. 2004) were used to amplify the *dsrA* gene. The amplicon size was ~220 bp. The SYBR Green I assay was always performed with a melting curve analysis to check PCR specificity. qPCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems) as follows: an initial incubation step for 2 min at 50°C (activation of the polymerase) followed by a 10 min pre-denaturation step at 95°C, and 40 cycles of denaturation for 30 s at 95°C and annealing for 1 min at 60°C. The PCR products from the environmental *dsrA* gene clones were ligated into pGEM T-Easy vector (Promega) and then used as the standard DNA for the *dsr* gene quantification (Cho et al. 2017).

Diversity of 16S rRNA genes and the *dsr* gene

The bacterial 16S rRNA genes obtained from the sediment samples collected at the 0–1 cm and 1–2 cm depths of the farm and reference sites were amplified using the primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Rho et al. 2005) and 1518R (5′-AAG GAG GTG ATC CAN CCR CA-3′) (Campbell et al. 2001) as described by Cho et al. (2017).

A ~1.9 kb *dsrAB* gene fragment obtained from the 1 to 2 cm depth of the sediments was amplified according to previously described PCR method (Harrison et al. 2009, Cho et al. 2017) using primers DSR1F (5′-ACS CAC TGG AAR CAC G-3′) (Wagner et al. 1998) and DSR4R-1 (5′-GTT ACC GCA RAA CAT RCA-3′).

PCR products were purified using the MEGA-quick-spin™ fragment DNA Purification kit (iNtRON Biotechnology). The purified PCR amplicons were ligated into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α cells. The insert DNA in the pGEM-T Easy vector was amplified using the primers M13F (-20) (5′- GTA AAA CGA CGG CCA G-3′) and M13R (-20) (5′-CAG GAA ACA GCT ATG AC-3′). All sequences obtained from SolGent (Daejeon, Korea) were checked for chimeric artifacts using the Pintail program of the Bioinformatics Toolkit (Ashelford et al. 2005). These sequences were aligned with closely related sequences obtained from GenBank (www.ncbi.nlm.nih.gov/BLAST) using ClustalW in MEGA 4.0. The filtered 16S rRNA and *dsrA* sequences that shared >97% similarity were grouped in the same operational taxonomic unit (OTU). Phylogenetic trees were constructed using the neighbor-joining method with the Jukes and Cantor distance model, implemented within MEGA 4.0. Node support was assessed by bootstrapping using 1000 bootstrap replicates.

Nucleotide sequence accession number

The bacterial 16S rRNA and *dsrA* sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers KC631436 to KC631612 for the bacterial 16S rRNA gene related to *Epsilonproteobacteria* and MH071538 to MH071597 for the *dsrA* gene.

Statistical analysis

The rarefraction analyses of partial sequences of 16S rRNA and *dsr* were calculated using the MOTHUR program (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/q010p413_supp.pdf; Schloss et al. 2009). To compare the difference of means for geochemical properties, POC, TN, SRR and the abundance of *dsr*A gene copy number, Student's *t*-test was used. Before analysis, the homogeneity of variance was checked using Levene's test.

RESULTS

Geochemical parameters

In the sediment trap, the suspended particulate matter (SPM), POC and TN at the farm site were ~2-fold higher than those of the reference site (Table 1). The POC and TN contents in the sediments and the acetic acid concentration in the porewater at the farm site were higher than those measured at the reference site, although the difference between the 2 sites was not significant ($p = 0.076$, $p = 0.096$ and $p = 0.149$, respectively, Table 1). The vertical profile of NH_4^+ in porewater increased with depth to 1424 µM at the farm site and to 1133 µM at the reference site (Fig. 2A). The average concentration of NH_4^+ down to 10 cm depth was 1.5-fold higher at the farm (1310 µM) than at the reference site (890 µM) (Table 2). NO_3^- concentrations were 5.2 µM and 3.8 µM at the sediment–water interface of the farm and reference sites, respectively, and decreased with depth to <2.4 µM at both sites (Fig. 2B). PO_4^{3-} concentration ranged from 7.70 to 17.56 µM at the

Table 1. Environmental parameters of the bottom water and surface sediments and vertical flux of particulate materials in the sediment trap at the farm and reference sites. DO: Dissolved oxygen; SPM: suspended particulate matter; POC: particulate organic carbon; TN: total nitrogen. Surface sediment values are mean (±1 SD) of 2 sediment cores and are down to 10 cm depth (for porosity and density) or 2 cm depth (POC, TN and acetic acid). Sediment trap data is from NIFS (2010)

	Lat./Long.	Bottom water				Sediment trap			Surface sediment				
		Water depth (m)	Temp. (°C)	Salinity (psu)	DO (mg l^{-1})	SPM flux (g m^{-2} d^{-1})	POC flux (g m^{-2} d^{-1})	TN flux (g m^{-2} d^{-1})	Porosity	Density (g cm^{-3})	POC (%, dry wt.)	TN (%, dry wt.)	Acetic acid (µM)
Farm	34° 34′ 37″ N/ 127° 41′ 06″ E	4.0	24	30.1	6.16	166	2.32	0.50	0.83 (± 0.03)	1.38 (± 0.04)	1.40 (± 0.01)	0.18 (± 0.01)	210 (± 0.03)
Reference	34° 34′ 38″ N/ 127° 41′ 06″ E	8.5	24	30.1	6.16	74.9	1.31	0.30	0.77 (± 0.03)	1.43 (± 0.06)	1.01 (± 0.05)	0.12 (± 0.01)	150 (± 0.04)

farm site and from 0.87 to 6.89 µM at the reference site (Fig. 2C). The average concentration of PO_4^{3-} was 3-fold higher at the farm site (10.74 µM) than at the reference site (3.74 µM). H_2S concentration in porewater was <8 µM down to 2 cm at the farm site but then rapidly increased with depth, reaching 394 µM at 10 cm, whereas the accumulation of H_2S was relatively indiscernible at the reference site (Fig. 2D). The average concentration of H_2S at the farm site (123.6 µM) was 15-fold higher than that at the reference site (8.20 µM) (Table 2). SO_4^{2-} concentrations were similar at both sites (~24 mM) (Fig. 2E). Fe^{2+} concentration ranged from 1.4 to 10.4 µM at the farm site and from 0.8 to 36.0 µM at the reference site (Fig. 2F). The average concentration of Fe^{2+} at the farm site (3.38 µM) was 6-fold lower than that at the reference site (20.31 µM). The average concentrations of NH_4^+, PO_4^{3-}, HS^- and Fe^{2+} at the farm site were significantly different from those measured at the reference site ($p < 0.0001$, $p = 0.006$, $p = 0.007$ and $p < 0.0001$, respectively), whereas the difference in concentrations of NO_3^- and SO_4^{2-} between the 2 sites was not significant ($p = 0.509$ and $p = 0.077$, respectively).

The total $Fe_{(oxal)}$ concentration ranged from 39.9 to 48.2 µmol cm^{-3} at the farm site and from 40.4 to 45.7 µmol cm^{-3} at the reference site (Fig. 2G,H). $Fe(III)_{(oxal)}$ comprised 50–94 % at the farm site and 50–99 % at the reference site (Fig. 2I). The average concentrations of $Fe(II)_{(oxal)}$ and $Fe(III)_{(oxal)}$ were similar at both the farm (17.39 Fe(II) µmol cm^{-3} and 26.58 Fe(III) µmol cm^{-3}) and reference sites (15.05 Fe(II) µmol cm^{-3} and 28.16 Fe(III) µmol cm^{-3}) (Table 2). The concentrations of $Fe(II)_{(oxal)}$ and $Fe(III)_{(oxal)}$ were not significantly different between the farm and reference sites ($p = 0.082$ and $p = 0.157$, respectively). Average concentrations of total reduced sulfur (TRS) ranged from 71.4 to 259 µmol cm^{-3} at the farm site and from 9.21 to 129 µmol cm^{-3} at the reference site (Fig. 2J), and the TRS concentration between the 2 sites was significantly different ($p = 0.0005$).

SRR and *dsr* gene copy number

SRR ranged from 753 to 1957 nmol cm^{-3} d^{-1} at the farm site and from 22.1 to 77.0 nmol cm^{-3} d^{-1} at the reference site (Fig. 3A). Average SRR at the farm site (1209 nmol cm^{-3} d^{-1}) was 19-fold higher than that measured at the reference site (62.1 nmol cm^{-3} d^{-1}). The abundance of *dsr* gene copy number ranged from 1.1×10^9 to 3.4×10^9 copies cm^{-3} at the farm site and from 4.3×10^8 to 1.3×10^9 copies cm^{-3} at the ref-

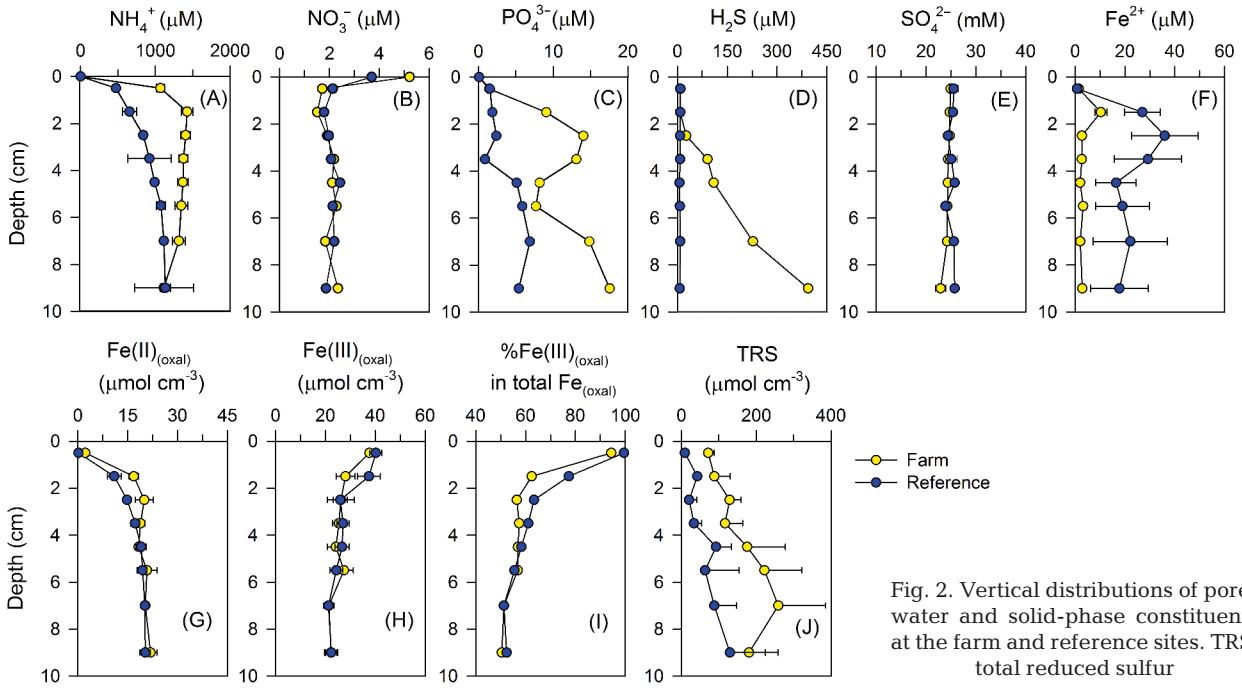

Fig. 2. Vertical distributions of porewater and solid-phase constituents at the farm and reference sites. TRS: total reduced sulfur

erence site (Fig. 3B). The peak of the *dsr* gene copy number (3.4×10^9 copies cm^{-3}) was observed at a depth of 1 to 2 cm where SRR was maximal (1958 nmol cm^{-3} d^{-1}). The SRR and the abundance of *dsr* gene copy number were significantly different between the farm and reference sites ($p < 0.0001$ and $p = 0.0001$, respectively).

Bacterial community composition

A total of 376 bacterial 16S rRNA gene sequences were analyzed to elucidate the major bacterial groups inhabiting the surface sediments at both sites. These sequences were assigned to 302 OTUs based on a 3% cutoff (Table S1 in the Supplement). The coverage rates of 16S rRNA gene libraries were 11 and 9% at a depth of 0–1 cm at the farm and reference sites, respectively, and 59 and 8% at a depth of 1–2 cm at the farm and reference sites, respectively (Table S1). Rarefaction curves based on the 16S rRNA gene sequences were obtained by plotting with the observed OTUs for each library (Fig. S1), yet none reached the curvilinear or plateau phase at the species level (3% difference) (Fig. S1). However, the underestimation of diversity at the family and order levels (10 and 16% difference) was less significant since the curves came close to reaching a plateau. Most sequences (>50% of total 16S rRNA gene sequences) were affiliated with the *Alpha-*, *Gamma-*, *Delta-* and *Epsilonproteobacteria*, *Acidobacteria* and several minor groups including *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Betaproteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus*, *Firmicutes*, *Fusobacteria*, *Parcubacteria*, *Planctomycetes* and *Verrucomicrobia* (see Fig. 6, Table S1). Major groups occupying >15% of total 16S rRNA gene sequences were related to *Gamma-*, *Delta-* and *Epsilonproteobacteria* (Table 3).

In total, 114 *dsrA* gene sequences were obtained from the 1–2 cm depth interval and were sorted into 70 OTUs using our definition of >97% sequence identity (Table S2). All *dsrA* gene sequences were affiliated with bacteria, and no archeal *dsrA* genes were detected. The coverage of the *dsrA* gene libraries showed 35 and 43% at the farm and reference sites, respectively. Rarefaction curves based on *dsrA* gene sequences at the farm and reference sites approached near saturation at the family level (10% difference) (Fig. S1).

Bacterial communities associated with sulfate reduction

Sulfate-reducing bacteria (SRB) revealed by 16S rRNA gene sequences were closely associated with *Desulfarculaceae*, *Desulfobulbaceae*, *Desulfuromonadaceae* and *Syntrophobacteraceae* in *Deltaproteobacteria* at both sites (Table 3). The relative abundance of SRB in *Deltaproteobacteria* comprised 11% (0–1 cm depth) and 6% (1–2 cm depth) of total 16S

Table 2. Concentrations (mean ± SD, n = 3) of inorganic nitrogen (NH_4^+, NO_3^-), PO_4^{3-}, dissolved H_2S, SO_4^{2-} and dissolved Fe^{2+} in porewater, and solid-phase $Fe_{(oxal)}$ and total reduced sulfur (TRS), sulfate reduction rate (SRR) and DNA copy number of the *dsrA* gene in the sediment of the farm and reference sites (*p < 0.01, **p < 0.001)

Site	Porewater (μM)					Solid-phase (μmol cm^{-3})					
	NH_4^{+**}	NO_3^-	PO_4^{3-*}	H_2S^*	SO_4^{2-}	Fe^{2+**}	Total $Fe_{(oxal)}$	$Fe(II)_{(oxal)}$	$Fe(III)_{(oxal)}$	TRS**	SRR** (nmol cm^{-3} d^{-1})	*dsrA* gene copy number** (DNA copies cm^{-3})
Farm	1310 (± 161)	1.99 (± 0.29)	10.74 (± 5.14)	123.6 (± 136)	24.33 (± 0.69)	3.38 (± 2.94)	43.99 (± 3.82)	17.39 (± 6.20)	26.58 (± 5.29)	149.4 (± 83.7)	1209 (± 380)	1.9 × 10^9 (± 7.5 ×10^8)
Reference	890.1 (± 243)	2.07 (± 0.20)	3.74 (± 2.32)	8.20 (± 1.9)	24.88 (± 0.67)	20.31 (± 13.9)	43.49 (± 5.59)	15.05 (± 6.67)	28.16 (± 6.96)	62.96 (± 67.0)	62.1 (± 27.6)	9.5 × 10^8 (± 3.0 ×10^8)

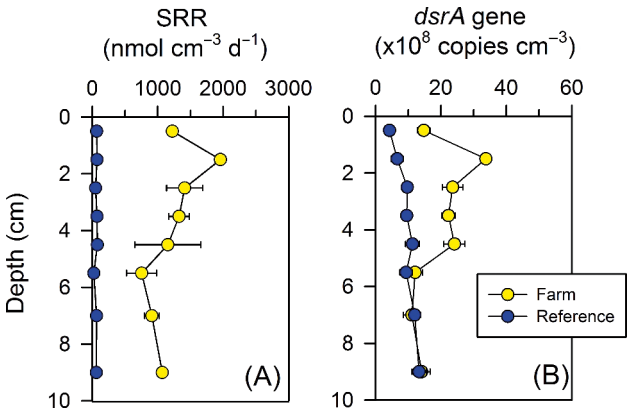

Fig. 3. Vertical distributions of (A) sulfate reduction rate (SRR) and (B) DNA copy number of the *dsrA* gene at farm and reference sites

rRNA gene sequences at the farm site and 10% (0–1 cm depth) and 28% (1–2 cm depth) of total 16S rRNA gene sequences at the reference site (Table 3). Particularly, *Desulfobulbus* (similarity > 95%) in *Desulfobulbaceae* appeared as the major member at both sites (Table 3).

Based on the *dsrA* functional gene analysis, 5 different phylogenetic groups were clustered (Fig. 4). SRB affiliated with *Syntrophobacteraceae* (Group III) appeared to be the predominant bacterial group at the farm and reference sites, comprising 57 and 53% of the total clones, respectively (Fig. 4, Table S2). Most sequences of this group were related to uncultured *Syntrophobacteraceae* clones discovered from the Aarhus Bay (JQ304781 and JQ304763; de Rezende et al. 2013) (similarity > 95%). However, they had low similarity (57–75%) with the cultured isolates (*Desulfacinum infernum*, *Desulforhabdus amnigena*, *Syntrophobacter wolinii*, *S. fumaroxidans* and *Thermodesulforhadus norvegica*) (Fig. 4, Table S2).

The second dominant SRB group (Group V), comprising 23 and 33% of total *dsrA* gene sequences at the farm and reference sites, respectively (Table S2), was not affiliated with any cultured SRB but clustered closely as a deep-branching lineage (Fig. 4). The deep-branching group in this study was associated with reported SRB lineage Group V encompassing Clone NTd-V07 (AB263178; Kaneko et al. 2007) (similarity 98%) in Nankai Trough deep-sea sediment and the DSR-F group including Clones TopDsr2, TopDsr35, TopDsr59, TopDsr78, MidDsr71 and BotDsr73 (FJ748832–FJ748843; Jiang et al. 2009) (similarity > 87%) in the Pearl River Estuary (Fig. 4, Table S2).

The next 2 minor groups, Group I and Group II, were closely related to *Desulfobacteraceae* and *Desulfobulbaceae*. The number of sequences affili-

Table 3. Bacterial composition and number of clones closely related to *Gamma-*, *Epsilon-* and *Deltaproteobacteria* found at the farm and reference sites. Numbers in parentheses represent the relative abundance of each clone. The putative function of closely related species (only sequence similarities >95%) is indicated. Chem O: chemoorganotrophy; F: fermentation; FeR: Fe(III) reduction; NR: nitrate reduction; SO: sulfur oxidation; SR: sulfate reduction. The total number of clones is the total number of bacterial 16S rRNA gene clones from Table S1

Phylogenetic group			No. of clones				Putative function
			Farm		Reference		
Class	Family	Genus	0–1 cm	1–2 cm	0–1 cm	1–2 cm	
Gammaproteobacteria	*Coxiellaceae*	*Coxiella*	1(1.0)				Unknown
	Ectothiorhodospiraceae	*Thioalbus*	2(2.0)	1(1.1)			SO
		Thioalkalivibrio	7(7.1)		5(4.7)	3(3.4)	SO
		Thiohalomonas	5(5.1)	2(2.2)	5(4.7)	3(3.4)	SO
	Halieacea	*Haliea*		1(1.1)		1(1.1)	NR
		Halioglobus	5(5.1)	3(3.3)	6(5.7)	3(3.4)	NR
	Oceanospirillales	*Marinomonas*	1(1.0)				Chem O
		Nitrincola	1(1.0)				NR
	Psychromonadaceae	*Psychromonas*			1(0.9)		F, NR
	Thioalkalispiraceae	*Endothiovibirio*			1(0.9)		SO
	–	*Thiohalobacter*	1(1.0)			1(1.1)	SO
	–	*Thiolapillus*	2(2.0)		1(0.9)		SO
	Thioprofundaceae	*Thioprofundum*	2(2.0)		1(0.9)		SO, NR
	Vibrionaceae	*Enterovibrio*			1(0.9)		F, NR
		Photobacterium		1(1.1)	3(2.8)		F, NR
		Vibrio			2(1.9)		Unknown
Epsilonproteobacteria	–	*Sulfurovum*	8(8.2)	57(62.0)	9(8.5)	7(7.9)	SO, NR
Number of total clones related to SO			27(27.6)	60(65.2)	22(20.8)	14(15.9)	
Deltaproteobacteria	*Desulfarculaceae*	*Desulfarculus*			1(0.9)		SR
	Desulfobulbaceae	*Desulfobacterium*	2(2.0)		2(1.9)	5(5.7)	SR
		Desulfobulbus	5(5.1)	3(3.3)	4(3.8)	12(13.6)	SR
		Desulfocapsa		1(1.1)			SR
		Desulfopila		1(1.1)	1(0.9)	1(1.1)	SR
		Desulfotalea	1(1.0)				SR
		Desulfurivibrio	1(1.0)	1(1.1)	1(0.9)	2(2.3)	SR
	Desulfuromonadaceae	*Desulfuromonas*	2(2.0)			1(1.1)	FeR, SR
		Pelobacter	1(1.0)		3(2.8)	1(1.1)	F
	Geobacteraceae	*Geobacter*	1(1.0)		1(0.9)		FeR
	Kofleriaceae	*Haliangium*			1(0.9)		SR
		Kofleria			1(0.9)		SR
	Sandaracinaceae	*Sandaracinus*	1(1.0)	1(1.1)	2(1.9)		F
	Syntrophaceae	*Syntrophus*	3(3.1)		1(0.9)		F
	Syntrophobacteraceae	*Syntrophobacter*				4(4.5)	F, SR
		Syntrophorhabdus		1(1.1)			F
Number of total clones related to SR			11(11.2)	6(6.5)	11(10.4)	25(28.4)	
Number of total clones			98	92	106	88	

ated with Group I accounted for 11 and 6% of total *dsrA* gene sequences at the farm and reference sites, respectively (Table S2). The sequences of Group I were associated with uncultured SRB bacterium clones detected from Aarhus Bay sediments (AM408827; Leloup et al. 2009, JQ304757; de Rezende et al. 2013) (similarity: 95%), Mediterranean Sea oligotrophic waters (FM212289; Giloteaux et al. 2010) (similarity: 95%) and Ulleung Basin sediments (JN798936; Cho et al. 2017) (similarity: 96%). Only clone YF2_23 at the farm site was closely related to *Desulfococcus multivorans*, with a similarity of 97% (Table S2). The sequences of Group II accounted for 8% of total *dsrA* gene sequences at the reference site (Table S2). Most sequences of Group II were closely related to those of uncultured SRB found in environmental samples from sites such as Victoria Harbor in Hong Kong (DQ112190; Zhang et al. 2008) (similarity > 96%) and a salt marsh on the east coast of the USA (KP992730; Angermeyer et al. 2016) (similarity: 90%); both sites are affected by high organic material input via anthropogenic activities (Fig. 4).

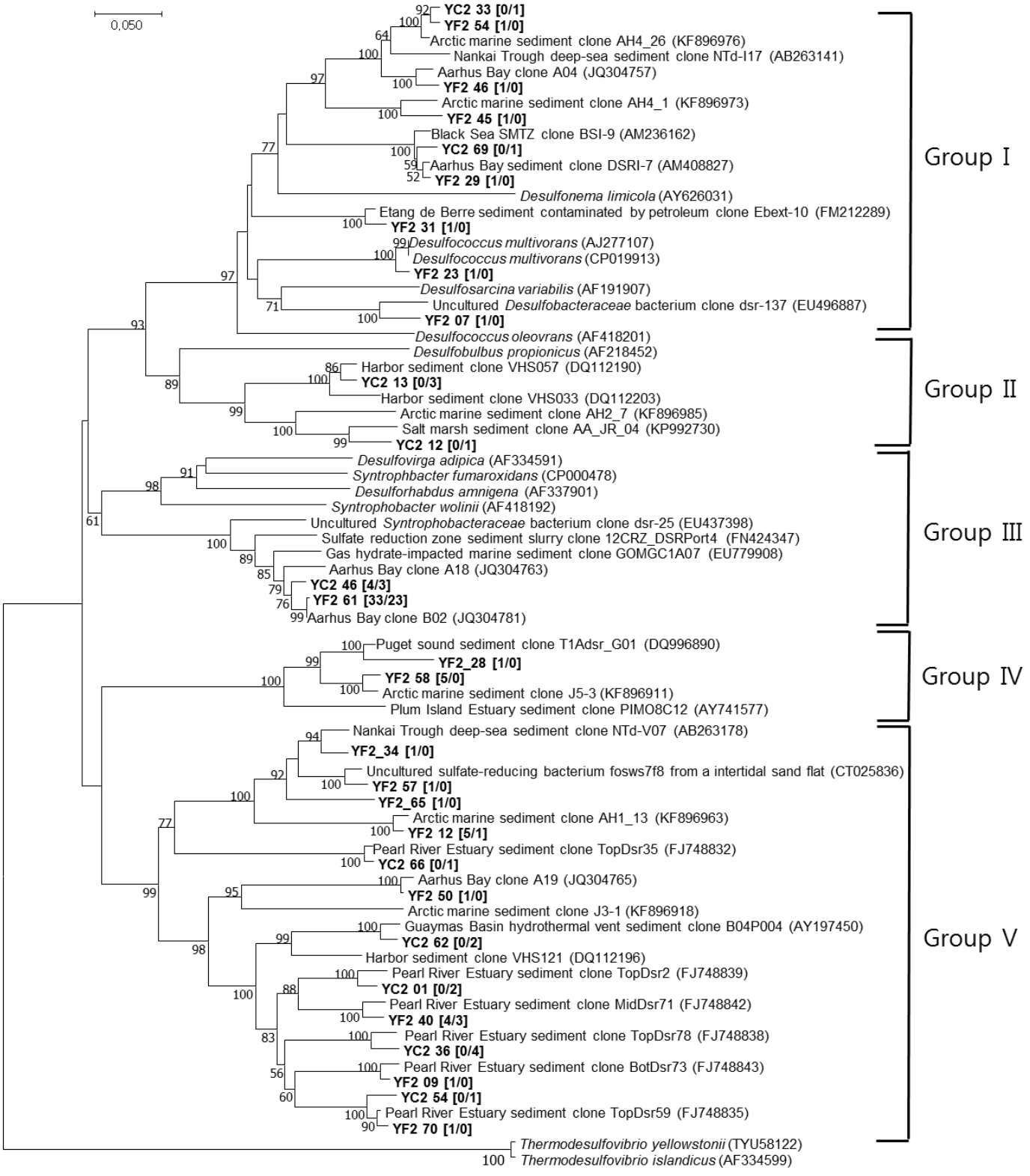

Fig. 4. Phylogenetic tree based on partial sequences of *dsrA* from sulfate-reducing prokaryotes, including partial sequences based on environmental *dsrA* gene amplicons. Sequences were retrieved from sediment (1–2 cm depth) of the farm (**YF**) and reference (**YC**). The tree was constructed using the neighbor-joining method with *Thermodesulfovibrio yellowstonii* and *T. islandicus*. Bootstrap values are based on 1000 replicates and are indicated at branch nodes for values >50% bootstrap support. Numbers in square brackets indicate the number of *dsrA* gene sequences detected from YF and YC. GenBank accession numbers for each sequence are indicated in parentheses

Bacterial communities associated with sulfur oxidation

As shown by the 16S rRNA gene sequencing, sulfur-oxidizing bacteria (SOB) were closely related to *Gamma-* and *Epsilonproteobacteria* (Table 3). The relative abundance of SOB affiliated with *Gammaproteobacteria* was 19% (0–1 cm depth) and 3% (1–2 cm depth) of total sequences at the farm site, whereas they accounted for 12% (0–1 cm depth) and 8% (1–2 cm depth) of total sequences at the reference site (Table 3). Members such as *Thioalbus*, *Thioalkalivibrio* and *Thiohalomonas* belonging to the family *Ectothiorhodospiraceae* (similarity > 90%) appeared as the dominant SOBs (Table 3). Known as denitrifying chemolithoautotrophic SOB, these bacteria have been isolated from various environments such as the East Sea, hydrothermal vents and hypersaline environments (Sorokin et al. 2001, Park et al. 2011, Nunoura et al. 2014).

Unlike the distribution of SOB related to *Gammaproteobacteria*, the relative abundance of the *Epsilonproteobacteria* in total clones increased dramatically from 8% at 0–1 cm depth to 62% at 1–2 cm depth at the farm site, while this group comprised 8% at both depths at the reference site (Table 3, Table S1). All sequences belonging to *Epsilonproteobacteria* were related to uncultured sequences detected in intertidal sediments (DQ112501), deep-sea sediments with substantial organic loading (GQ 261802; Goffredi & Orphan 2010), a deep-sea mud volcano (HQ588432; Pachiadaki et al. 2011) and mariculture sediments (JX193374; Li et al. 2013) with similarity > 93% (Fig. 5). The epsilonproteobacterial sequences were closely affiliated with *Sulfurovum lithotrophicum* (similarity > 95%) and *S. riftiae* (similarity > 94%), chemolithoautotrophs which use S^0 or $S_2O_3^2$ as an electron donor and O_2 or NO_3^- as an electron acceptor (Inagaki et al. 2004, Giovannelli et al. 2016) (Fig. 5).

DISCUSSION

Impact of the fish farm on sediment geochemistry and sulfur cycles

The fish-farm sediments were characterized by highly reduced conditions with increased accumulation of NH_4^+, PO_4^{3-} and H_2S (Fig. 2, Table 2) and extremely high SRR compared to measurements at the reference site (Fig. 3). The SRRs reported here were within the range (92 to 310 mmol m^{-2} d^{-1}) reported from other organic-rich fish-farm sediments (Holmer & Kristensen 1992, Holmer et al. 2003, 2005) but were markedly higher than those measured in shellfish farms (30–61 mmol m^{-2} d^{-1}), where no artificial fish feed is used (Dahlbäck & Gunnarsson 1981, Holmer et al. 2003, Kim et al. 2011, Hyun et al. 2013).

The most intriguing geochemical property observed in the fish-farm sediment was the sulfide depletion at the 0 to 2 cm depth (Fig. 2D), where the highest SRR and *dsr* gene abundance were also observed (Fig. 3). A combination of microbial C_{org} oxidation by Fe(III) reduction and abiotic reduction of Fe(III) coupled with S oxidation is responsible for this mismatch of sulfide (Thamdrup et al. 1993, Canfield et al. 2005). First, the amounts of $Fe(III)_{(oxal)}$ (28–37 µmol cm^{-3}) appeared to be a major solid form of $Fe_{(oxal)}$, comprising 60 to 95% of total $Fe_{(oxal)}$ at the 0–2 cm depth in the fish-farm sediments (Fig. 2H,I). The availability of $Fe(III)_{(oxal)}$ ultimately stimulates microbial C_{org} oxidation coupled with Fe(III) reduction, thereby resulting in an accumulation of Fe^{2+} (Eq. 1; Canfield et al. 2005). Second, the $Fe(III)_{(oxal)}$ in the sulfidic sediments is readily reduced by the sulfide to form Fe^{2+} and S^0 in the sediment (Eq. 2; Canfield & Thamdrup 1996). Both biotic and abiotic reduction of Fe(III) via Eqs. (1) and (2), respectively, should produce substantial amounts of Fe^{2+} in the surface sediment (Fig. 2F). Finally, at the farm site where sulfate reduction was high (Fig. 3A), the Fe^{2+} removed the sulfide to form FeS (Eq. 3; Canfield et al. 2005). Because the dissolved Fe^{2+} was highly depleted at the farm site (Fig. 2F), H_2S oxidation coupled with Fe^{2+} oxidation (Eq. 3) is likely to be higher at the farm site than at the reference site. Accordingly, average concentrations of TRS (H_2S, S^0, FeS and FeS_2) at the 0–2 cm depth interval were 3-fold higher at the farm site (80 ± 8.3 µmol cm^{-3}) than at the reference site (26 ± 17 µmol cm^{-3}) (Fig. 2J).

$$CH_3COO^- + 8FeOOH + 3H_2O = 2HCO_3^- + 8Fe^{2+} + 15OH^- \quad (1)$$

$$3H_2S + 4FeOOH = S^0 + S_2O_3^{2-} + 4Fe^{2+} + 5H_2O \quad (2)$$

$$H_2S + Fe^{2+} = FeS + 2H^+ \quad (3)$$

Bacterial communities associated with production and removal of reduced sulfur

Phylogenetic analysis of the *dsrA* functional gene and the 16S rRNA gene revealed that the sequences closely affiliated with *Syntrophobacteraceae* and *Desulfobulbaceae* predominated at both sites (Fig. 4, Table 3). Most members (e.g. *Desulfacinum*, *Desulforhabdus* and *Thermodesulforhadus*) in family *Syn-*

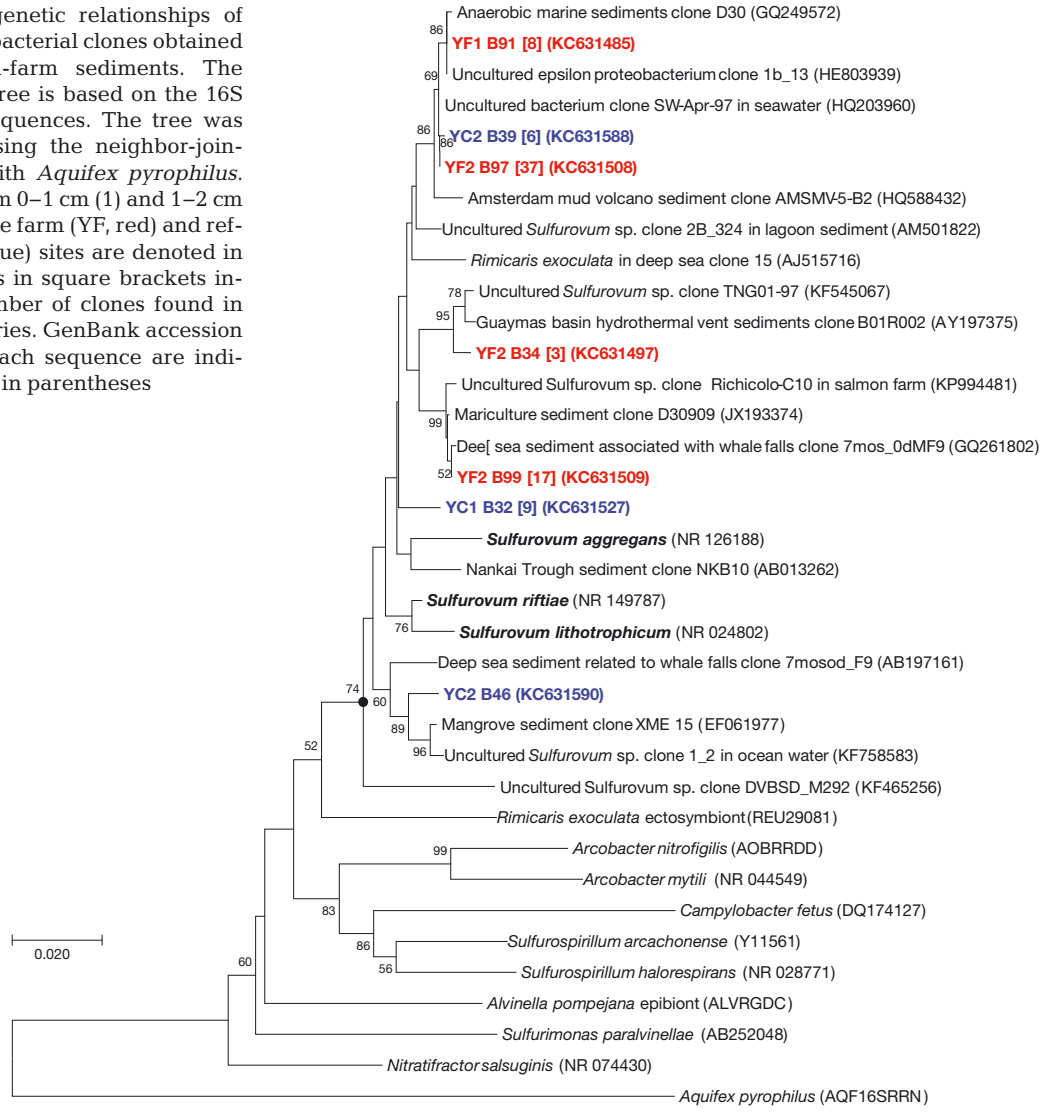

Fig. 5. Phylogenetic relationships of epsilonproteobacterial clones obtained from the fish-farm sediments. The phylogenetic tree is based on the 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method with *Aquifex pyrophilus*. The clones from 0–1 cm (1) and 1–2 cm (2) depths at the farm (YF, red) and reference (YC, blue) sites are denoted in **bold**. Numbers in square brackets indicate the number of clones found in the clone libraries. GenBank accession numbers for each sequence are indicated in parentheses

trophobacteraceae perform a complete oxidation of organic substrates, whereas the members of *Syntrophobacter* are known to be associated with an incomplete oxidation to produce acetate (Rosenberg et al. 2014). Due to the low similarity (57–75%) with the cultured isolates in Fig. 4, it remains to be resolved if the uncultured *Syntrophobacteraceae* groups obtained in our *dsrA* gene analysis (Fig. 4) are directly responsible for the complete or incomplete oxidation of C_{org}. However, the clones from the 16S rRNA gene sequence showed high similarity (> 95%) with *Desulfobulbus* (Table 3), which is known to oxidize a broad range of substrates (e.g. propionate, alcohols and lactate) resulting from various fermentation pathways to produce acetate in anoxic freshwater and marine sediments (Lien et al. 1998, Pagani et al. 2011, Sorokin et al. 2012, Rosenberg et al. 2014). Because incomplete oxidizers can grow faster than complete oxidizers in natural sediments receiving pulses of rich organic material (Widdel 1988, also see Canfield et al. 2005), the *Desulfobulbus*-like bacterial communities in this study seem to be able to proliferate readily around the fish-farm sediments.

Despite the high SRR and *dsrA* abundance, the depletion of sulfide at the 0–2 cm depth at the fish farm (Fig. 3A,B) remains to be explained in terms of which microorganisms are associated with the removal of sulfide. Interestingly, at the 1–2 cm depth interval of this sulfide mismatch layer, *Epsilonproteobacteria* closely affiliated with *Sulfurovum lithotrophicum* (similarity > 95%), *S. riftiae* (similarity > 94%) and *S. aggregans* (similarity > 95%) composed the major fraction (62.0%) of total sequences (Figs. 5 & 6, Table 3). Similar results were reported in the

sediments of salmon farms where *Epsilonproteobacteria* is a major bacterial group (Aranda et al. 2015), although the percentage (39% of total clones) was lower than that reported in the present study. Both *S. lithotrophicum* and *S. riftiae*, isolated from the oxic–anoxic interface where sulfides meet oxygenated sea water, and the vent polychaete *Riftia pachptila*, respectively, are known to be chemolithoautotrophs using S^0 or $S_2O_3^{2-}$ as an electron donor and O_2 or NO_3^- as an electron acceptor (Inagaki et al. 2004, Giovannelli et al. 2016) (Fig. 5). In this study, it is likely that the S^0 or $S_2O_3^{2-}$ was produced by the oxidation of H_2S coupled with the reduction of Fe(III) via Eq. (2) and served as electron donors for the *Sulfurovum*-like SOB that flourished in the sulfide-mismatched zone where either O_2 or NO_3^- is available as an electron acceptor. Unlike *S. lithotrophicum* and *S. riftiae*, *S. aggregans* uses H_2 as an electron donor and S^0, $S_2O_3^{2-}$ and NO_3^- as electron acceptors under more anoxic conditions (Mino et al. 2014). Thus, bacteria closely related to *S. aggregans* may play a significant role in the reduction of S^0 and $S_2O_3^{2-}$ in the 1–2 cm depth of fish-farm sediments.

In contrast to the *Sulfurovum*-like bacteria that thrived at the 1–2 cm depth, the clones that had high similarity (>95%) with *Thioalbus*, *Thioalkalivibrio*, *Thioalomonas*, *Thiohalobacter*, *Thiolapillus* and *Thioprofundum* in *Gammaproteobacteria* occupied a major fraction (12–19% of total clones) mostly at the 0 to 1 cm depth interval of both farm and reference sites (Fig. 6, Table 3). Both *Sulfurovum* and the members of *Gammaproteobacteria* are microaerophilic chemolithoautotrophic sulfur oxidizers (Sorokin et al. 2001, Park et al. 2011, Nunoura et al. 2014). *Gammaproteobacteria* have a kinetically advantageous energy-producing pathway when oxygen and reduced sulfur compounds are steadily supplied, whereas *Epsilonproteobacteria* have the metabolic versatility to adapt to transient environmental conditions where the shift from aerobic to anaerobic microbial communities oc-

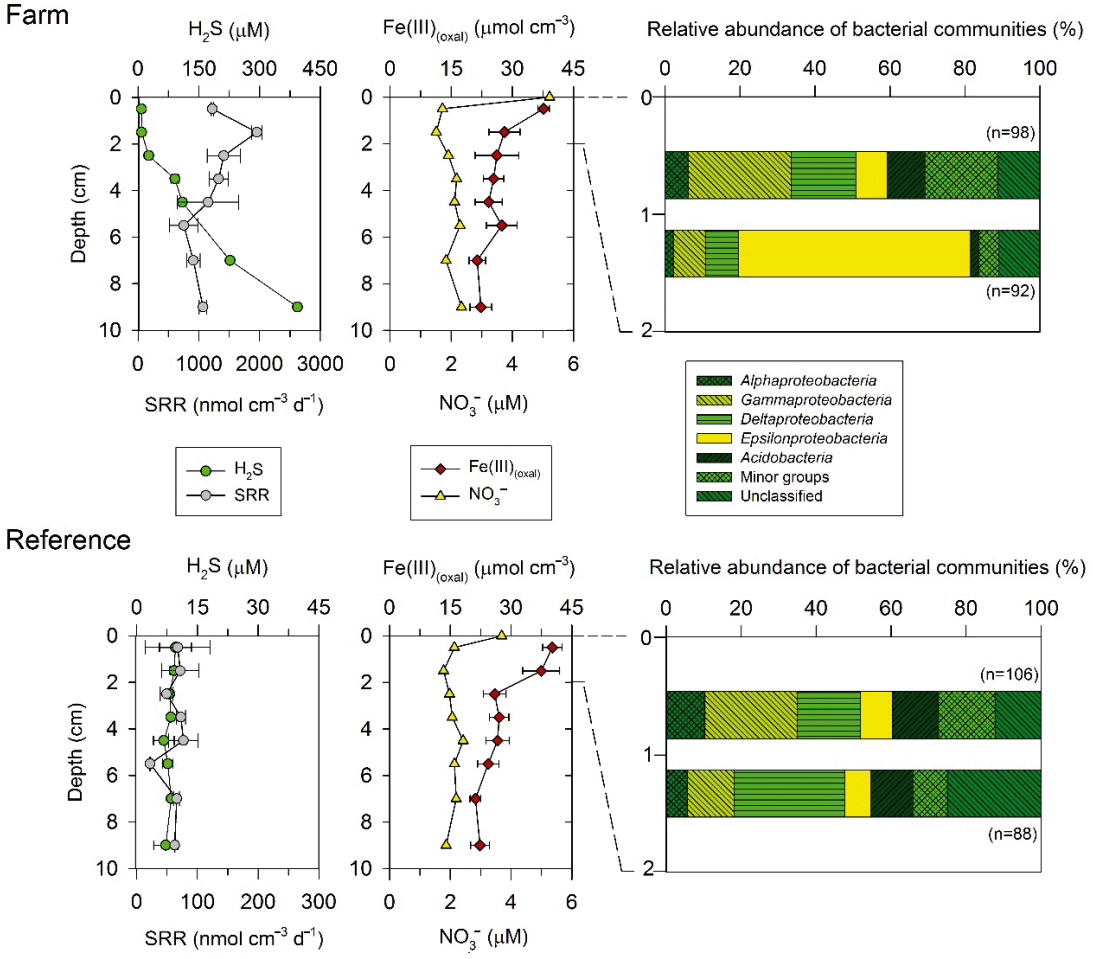

Fig. 6. Relative abundance of bacterial communities based on the 16S rRNA gene in the surface sediments (0–2 cm) associated with the vertical distribution of H_2S, NO_3^-, $Fe(III)_{(oxal)}$ and sulfate reduction rates (SRR). Bar represents the percentage of clone library composition represented by each group at the farm and reference sites

curs (Yamamoto & Takai 2011, Ihara et al. 2017). Consequently, our results strongly suggest that *Sulfurovum*-like bacteria in *Epsilonproteobacteria* play a significant ecological and biogeochemical role in the oxidation and reduction of reduced sulfur compounds in the highly sulfidic fish-farm sediments.

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