Aquaculture is of major industrial importance worldwide and this has led to concerns over the impact of fish farming on previously pristine marine environments. Fish farm sediments receive a large amount of organic matter due to uneaten food and fecal material. This organic enrichment of sediments influences the biogeochemical processes and benthic microbial communities (Asami et al. 2005, Holmer & Kristensen 1996, McCaig et al. 1999); and anaerobic conditions can develop resulting in the accumulation of hydrogen sulfide, ammonia, and methane. As a reductant, hydrogen sulfide traps oxygen and is converted to several sulfur oxianions (Bak et al. 1993, Jørgensen 1990a, 1990b, 1994, Jørgensen & Bak 1991, Luther et al. 1986, Wentzien et al. 1994). In eutrophic water, dissolved oxygen (DO) in the overlying water is deficient due to hydrogen sulfide production in the bottom sediment. Hydrogen sulfide is toxic to plants, animals and humans. Hydrogen sulfide in marine environments is mainly produced by dissimilatory reduction of sulfate by sulfate-reducing bacteria (SRB). Consequently, it is impossible to detect and enumerate all SRB species simultaneously with selective media and culture conditions. The dissimilatory sulfite reductase (DSR) gene sequence among the SRB is conserved; therefore, it is possible to design specific primers or probes for this enzyme (Karkhoff-Schweizer et al. 1995, Wagner et al. 1998). Kondo et al. (2004) developed PCR primers selective for the gene coding for the \( \alpha \)-subunit of DSR (\( \text{dsrA} \)) of most mesophilic SRB belonging to the \( \delta \)-Proteobacteria and used a quantitative competitive PCR to rapidly and reproducibly detect and count SRB, as an alternative to the culture-dependent method. This primer set was applied to enumerate SRB in various environments (Kondo et al. 2006, 2007, Kondo & Butani 2007, Leloup et al. 2007, Schippers & Neretin 2006).

Dissimilatory sulfate reduction by SRB is responsible for the production and accumulation of sulfide in marine sediments. Holmer & Kristensen (1996) showed stimulation of in situ sulfate reduction rates occurred in surface sediments of a marine fish farm in Kolding Fjord, Denmark. Asami et al. (2005) detected differences in the number and activities of sulfate reducers and sulfur oxidizers in two contrasting sediments in semi-enclosed bays on the Sanriku coast in Japan. They sug-
gested that these bacteria can serve as indicators for assessing the organic load to marine sediment. Despite the importance of SRB in fish farm sediments, little is known about their distribution with reference to organic enrichment in said sediments. A study was conducted by Kimata et al. (1955b) who reported their distribution in the sediments of Hiroshima Bay, Japan along an organic gradient, according to enumeration using the MPN method. They showed a correlation between SRB numbers and the sulfide and organic content in the sediments. Several studies, however, have demonstrated that there are no significant differences in SRB populations between marine sediment samples with different organic and/or sulfide contents (Ishida 1982, Kondo 1992). This may be due to inexact counting of SRB by culture-dependent methods.

The aim of this study was to investigate the distribution of SRB in sediments under fish cages for torafugu (Takifugu rubripes Temminck & Schlegel 1850) aquaculture along the coast of southern Fukui Prefecture, Japan, using a quantitative competitive PCR targeting the \textit{dsrA} genes and to relate this to pollution levels. We discovered differences in SRB densities in fish farm sediments with different levels of organic pollution.

Sediment samples were collected from April 2004 to November 2006 at four sites (Ano, Nyu, Hibiki and Te) along the coast of Wakasa Bay in southern Fukui Prefecture. The surface sediment was collected using an Ekman-Birge type bottom sampler (Rigo-sha) and sub-samples were taken from the 0–4 cm depth horizon using a syringe with the needle end cut-off. Samples for sulfide analysis were fixed with zinc acetate powder immediately after sampling. The samples were transported on ice to the laboratory.

The total sulfides (TSs) in the sediments were separated using steam distillation under acid conditions and trapped in a 10% (w/v) zinc acetate solution. The trapped sulfides were determined spectrophotometrically using the methylene-blue method (Kondo 2000). Chemical oxygen demand (COD) was measured using the standard Mn-COD method (Eguchi 2000). Moisture content was determined by drying the sediment samples at 60°C until a constant weight was obtained.

Total nucleic acids were extracted from a 0.5-g sediment sample using the hydroxyapatite spin-column method (Purdy et al. 1996). After the final ethanol precipitation, the nucleic acids were resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Nucleic acid purity and yield were determined using scanning spectrophotometry (Sambrook et al. 1989). One microliter of the DNA extract was diluted ten-fold with TE and used for PCR. Competitive PCR was performed as described in earlier work (Kondo et al. 2004, Kondo & Butani 2007) to determine the distribution of the SRB in the sediments.

Table 1 shows the COD and TS content in the sediment at each sampling site. COD and TS content in the sediments varied seasonally. Both COD and TS content in the sediments at Nyu were significantly higher than those at the other three sites (t-test, \( p < 0.01 \)). The data indicate organic enrichment and anoxic conditions may have developed in the organic rich Nyu sediments to a greater extent than at the other three sites. According to the environmental standards for aquaculture (Japan Fisheries Resources Conservation Association 2005), the Nyu sediments were classified as “polluted sediments” and the sediments at the other three sites were “normal sediments.”

We obtained fish farm sediments with different pollution levels, or organic contamination and sulfide contents. We determined the distribution of SRB using PCR in the same sediment samples as used for the chemical analyses. SRB were detected by competitive PCR in all four sediments at 4.9×10^{7} to 4.4×10^{9} cells g^{-1} dry sediment (Fig. 1) which is within the

<table>
<thead>
<tr>
<th>Study sites</th>
<th>Longitude (E)</th>
<th>Latitude (N)</th>
<th>COD (mg O_2 g^{-1} dry sediment)</th>
<th>TS (mg S g^{-1} dry sediment)</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hibiki</td>
<td>135°28’</td>
<td>35°32’</td>
<td>4.86–9.01</td>
<td>0.00–0.01</td>
<td>5</td>
</tr>
<tr>
<td>Ano</td>
<td>135°47’</td>
<td>35°32’</td>
<td>1.78–5.21</td>
<td>0.00–0.16</td>
<td>8</td>
</tr>
<tr>
<td>Nyu</td>
<td>135°57’</td>
<td>35°42’</td>
<td>14.61–40.45</td>
<td>0.28–0.96</td>
<td>8</td>
</tr>
<tr>
<td>Te</td>
<td>136°02’</td>
<td>35°42’</td>
<td>1.78–3.88</td>
<td>0.01–0.04</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 1. Relationship between sulfate-reducing bacterial (SRB) cell density determined by competitive PCR and chemical oxygen demand (COD; filled symbols) or total sulfide (TS; open symbols) in the sediments at Hibiki (triangle), Ano (square), Nyu (circle), and Te (diamond).
range of or slightly higher than values reported for other marine sediments as estimated using quantitative PCR with the same primer sets previously (Kondo et al. 2004, Leloup et al. 2007, Schippers & Neretin 2006). Our previous studies on the distribution of SRB in marine sediments used culture-dependent methods to enumerate SRB. Several studies, however, demonstrate that numbers of viable SRB in aquatic sediments are underestimated by a factor of more than a thousand when standard MPN methods are used with selective enrichment culture media (Gibson et al. 1987, Jørgensen 1978, Kondo 1992). Our competitive PCR may be a more reliable method to enumerate most SRB in nature because the cell-specific rates of sulfate reduction are calculated using SRB cell counts with competitive PCR and at the same time we measured the in situ sulfate reduction rates, and these were within the range of, or lower than those of pure cultures of SRB (Kondo et al. 2004).

Even in the “normal sediments” of Ano, Te, and Hibiki, high densities of SRB ranging from 4.9×10^9 cells g^-1 dry sediments to 8.3×10^9 cells g^-1 dry sediment were detected using the competitive PCR. The estimated SRB population at Ano fluctuated 17-fold during our investigation, while at Nyu, Hibiki, and Te, the populations fluctuated by 5-, 4-, and 3-fold, respectively. Abdollahi & Nedwell (1979) investigated colony count of SRB in a saltmarsh sediment and concluded that temperature did not appear to alter the SRB population. The water temperature at 1 m above the bottom ranged from about 10°C to 28°C during our investigation, irrespective of sampling sites. The cell densities did not show any distinct seasonal fluctuation, suggesting that temperature may not be an important factor affecting the SRB population in the fish farm sediments investigated. Using the competitive PCR for enumeration of SRB, it was demonstrated that the SRB in the sediment of Colne estuary, UK, were abundant and showed no distinct seasonal or spatial fluctuations (Kondo et al. 2004). Total numbers of viable SRB in bottom sediments of estuarine environments have been reported to be little influenced by the organic content, sulfate content or salinity of the sediment (Kimata et al. 1955a). At Ano, fluctuations in COD and TS were minimal (Table 1), and the large fluctuations in the estimated number of SRB may have been caused by other environmental factors such as E_h or sediment porosity. Thus the SRB population may not be affected by organic content in sediments with low levels of organic pollution.

The relationship between the SRB cell densities and COD or TS content is shown in Fig. 1. In the “normal sediments” (COD less than 20 mgO_2 g^-1 dry sediment and TS less than 0.2 mgS_2 g^-1 dry sediment) at Ano, Hibiki, and Te, SRB densities did not exceed 10^9 cells g^-1 dry sediment. In contrast to the “normal sediments”, high densities of SRB at more than 10^9 cells g^-1 dry sediments were detected in the “polluted sediments” with high organic and TS content at Nyu except for in one sample. The densities were significantly higher than those in the sediments at the other three sites (t-test, p<0.05). Holmer & Kristensen (1996) reported that stimulation of sulfate reduction rates occurred in the surface layers of marine fish farm sediments where organic matter was deposited. An other study that analyzed changes in the composition of bacterial assemblages and activities in marine sediments in response to shellfish aquaculture (Asami et al. 2005) demonstrated differences in the number and activity of SRB and sulfur-oxidizing bacteria in two different coastal bays in the Sanriku area, Japan. These results suggest that SRB may be positively selected for within polluted fish farm sediments and that the abundance and activity of SRB are influenced by the amount of pollution. Here, we detected differences in SRB densities in fish farm sediments with different levels of organic pollution, suggesting SRB cell density may be used as a biological indicator to assess pollution levels in sediments of marine fish farms as an adjunct to chemical analysis such as COD and TS, which are commonly employed as environmental indicators for aquaculture (Japan Fisheries Resources Conservation Association 2005). To support this idea, further surveys should be performed where sediment samples from coastal sites with differing pollution levels are analyzed using the same methods introduced in the present study.

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Reference

SRB in fish farm sediments


