Effect of Magnesium Peroxide Biostimulation of Fish Feed-Loaded Marine Sediments on Changes in the Bacterial Community

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The effect of an oxygen-releasing compound (ORC) magnesium peroxide (MgO2) on the changes in the bacterial community in organically polluted sediment of aquaculture farms was tested in a microcosm experiment. The sediment, to which fish feed was added, was treated with 1% or 5% MgO2. The addition of fish feed induced a highly reduced environment with low redox potential, high total sulfides, and abundance of sulfate-reducing bacteria (SRB). Although the sediment remained highly reduced at 1% MgO2, there was a significant reduction of total sulfides, increase of redox potential, and resultant reduction of SRB. The bacterial community clearly changed with the treatments according to denaturing gradient gel electrophoresis (DGGE) analysis of 16S ribosomal RNA gene (16S rDNA). Aerobes disappeared in the fish feed-added sediment, and some SRB emerged in place of these aerobes. On the other hand, the SRB disappeared in the ORC-amended sediment due to its highly oxic condition. This study revealed the bacterial community in the sediments was affected mainly by the redox potential and resultant sulfides produced by SRB, but total organic carbon and nitrogen were not determinants of the microbial population.

Key words: Aquaculture / Bacterial community shift / Biostimulation / Magnesium peroxide / Oxygen-releasing compound.

INTRODUCTION

For the past decades, coastal eutrophication caused by aquaculture has threatened sensitive neighboring ecosystems such as coral reefs (Arboleda and Reichardt, 2009; Garren et al., 2008; Loya et al., 2004; Villanueva et al., 2006), seagrass beds (Apostolaki et al., 2007; Cancemi et al., 2003; Ruiz et al., 2001), and benthic faunas (Hargrave et al., 2008; Mazzola et al., 2000; Nickell et al., 2003; Santander-De Leon et al., 2010; Sanz-Lazaro and Marin, 2006). Maintenance of these ecosystems with active fish farms within the allowable sulfide concentration and redox potential remains to be a challenge. Thus, recent studies have focused on the bioremediation of the polluted fish farm vicinities.

Bioremediation is defined as the biological degradation of the organic matter contaminant (Romantschuk et al., 2000). However, in the fish farms, organic matter degradation becomes rate-
limiting once anaerobic processes become dominant (Reichardt et al., 2007; Reichardt et al., 2011). It was previously suggested that organic matter degradation is reduced by oxygen depletion and high sulfide conditions (Holmer, 1999; Hoppe et al., 1990; Hulthe et al., 1998; Kristensen, 1995). Oxic conditions enhance faster organic matter degradation of refractory material adsorbed to mineral surfaces within the sedimentary mesopores. Usually, the buried materials are adsorbed into the mesopores, resulting in less exposure to exoenzymatic degradation (Kristensen, 1995). However, the capacity of the aerobic microbial community to produce H₂O₂ diffuses the adsorbed materials into the mesopores and induces their hydrolysis (Hulthe et al., 1998).

Biostimulation, such as the use of oxygen-releasing compounds (ORC), is commonly introduced to enhance organic matter degradation and cycling in anaerobic conditions beneath fish farms (Vezzulli et al., 2004). Magnesium peroxide (MgO₂) is one of the representatives: it reacts with H₂O to release molecular oxygen. The chemical equation is:

\[ 2\text{MgO}_2 + 4\text{H}_2\text{O} \rightarrow 2\text{H}_2\text{O}_2 + 2\text{Mg(OH)}_2 \]

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

Despite the popular use of MgO₂ as ORC in petroleum-contaminated soil and groundwater aquifers, there is limited knowledge on its applicability to organically enriched aquaculture vicinities. Vezzulli et al. (2004) studied the effect of MgO₂ intercalated with phosphate in fish cage sediments, which showed significant enhancement of microbial degradation efficiency. This result suggests a positive effect on the bacterial activity; however, changes in bacterial community dynamics which reflect biogeochemical processes during biostimulation have yet to be determined. Moreover, there is paucity of knowledge on the microbial community of sediments influenced by fish farms.

In the present study, in order to determine the effect of MgO₂ as a biostimulation agent, a microcosm simulating the sediment of aquaculture farms with a high load of organic matter was made, and redox potential, sulfide concentration, dominance of sulfate-reducing bacteria (SRB), and amounts of organic carbon and nitrogen were estimated as indicators of the sediment condition. In addition, bacterial communities among non-polluted, polluted, and ORC-remediated sediments were compared with a molecular fingerprinting technique.

**MATERIALS AND METHODS**

**Sediment source**

Sediment was haphazardly collected from Lake Shiraiishi, a brackish water lake (34°6’ 35°-34°6’ 54”N; 136°14’ 4”E) in Kihoku, Mie, Japan, with an Ekman Birge sampler. The surface layer, whose depth was 0-5 cm, was sliced, pooled and equilibrated at 25°C for 7 days.

**Microcosm setup**

The equilibrated sediment at a volume of 20.36 cm³ was transferred into an autoclaved tightly sealed 50 mL container (3.24 cm internal diameter, the sediment was 0.62 cm in thickness) and allowed to settle under 25°C incubation for 7 days. To simulate fish farm waste sedimentation, mashed artificial Japanese flounder fish feed pellets (Higashimaru Corp., Kagoshima, Japan) was added on the surface of the sediment at a concentration of 250 g m⁻² with 5 mL autoclaved, 0.2 μm-filtered natural seawater to avoid desiccation, while a microcosm without the addition of feed was prepared as a negative control. The feed contained 50% crude protein, 8% crude fat, 2% crude fiber, 16% crude ash, 2% calcium, and 1.5% phosphorus based on the manufacturer’s information. After 20-day incubation of the setups, magnesium peroxide (MgO₂) was added to the feed-added sediment at a final concentration of 1% or 5% (w/v), which was approximately 1/10 or 1/2 of the concentration used by Vezzulli et al. (2004). The rest of the feed-added setups were kept without any addition of MgO₂ as a positive control. MgO₂ was mixed with the sediment for 1 min, and the mixtures were incubated at 25°C for 30 more days. In the following sections, the microcosm setups are as follows: Control, untreated sediment (a negative control); Feed, sediment to which fish feed was added (a positive control); 1% MgO₂, the same as Feed, but 1% MgO₂ was added after a 20-day incubation; 5% MgO₂, the same as 1% MgO₂, but the concentration of MgO₂ was 5%. The microcosm sediments were sampled after a 50-day incubation for chemical variable analyses as well as microbial DNA extraction.

**Chemical Variables of the sediments**

Moisture contents of the sediments were measured after being dried overnight at 100°C. pH and redox potential (Eh) were measured with a pH/Eh meter (PRN-41, Fujiwara Co., Tokyo, Japan).

For total organic carbon (TOC) and total organic nitrogen (TON) analyses, the sediments were incubated at 60°C until they dried up. Around 4 g of the dried sediments were treated with 5 mL 2 M HCl until carbon dioxide gas bubbles disappeared in order to remove inorganic carbon. The sediments were rinsed with distilled water thrice by shaking and centrifuging, and were re-dried at 60°C. The sediment samples of
1.2 mg each were combusted at 900°C (Hedges and Stern, 1984) and then analyzed by a CHNS analyzer Flash EA112 (Thermo Fisher Scientific, Massachusetts, USA). Total sulfides in the 0.25 g sediment samples were measured by using a colorimetric tube detection kit (Gastec Co., Kanagawa, Japan) based on the manufacturer’s instruction (Asami et al., 2005; Yamamoto et al., 1998). Values of TOC, TON and total sulfides are shown all throughout the manuscript on a dry weight basis.

**Enumeration of sulfate-reducing bacteria**

Sulfate-reducing bacteria (SRB) were counted by the most probable number (MPN) method using Medium B according to Postgate (1979). Briefly, the sediments were suspended in sterilized natural seawater and serially diluted by ten-fold. Three hundred μL of the diluted solutions were inoculated into Medium B semi-solidified by agar, placed into 24 well microplates, and cultivated at 25°C for 14 days in the anaerobic bacteria culture kit, Anaeropack Kenki (As One, Osaka, Japan). Wells that became black by SRB growth and resultant ferric sulfide production were counted, and the cell numbers were calculated according to the MPN method.

**Bacterial community analyses by denaturing gradient gel electrophoresis**

Microbial DNA was extracted from 0.5 g of the sediment samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The 16S ribosomal RNA gene (16S rDNA) was amplified using a touch-down PCR with thermal cycling conditions as follows: initial denaturation of 95°C for 1 min, 19 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1 min with a decreasing temperature of -0.8°C at every cycle, and extension at 72°C for 1 min, followed by 9 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR primers used were -357F (5'-CCTACGGAGGCAGCAG-3') with GC clamp (Muyzer et al., 1993) and 907r (5'-CCGCTACACGAGGAGGC-3') (Yu and Morrison, 2004). The PCR reaction mixture at a volume of 250 μL was composed of 6.25 μL of 10-times-diluted microbial DNA, ExTaq buffer, 100 μM each dNTP mixture, 0.5 μM of the forward and reverse primers, and 0.025 units μL⁻¹ ExTaq DNA polymerase (Hot Start Version, Takara Bio, Otsu, Japan). Each PCR was run in 5 tubes of 50 μL. PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega Corp., Madison, WI, USA).

Amplified and purified 16S rDNA fragments were run in denaturing gradient gel electrophoresis (DGGE) with D-Code System (Bio-Rad, Hercules, CA, USA) according to Ishii et al. (2000). Approximately 2.8 μg of the purified DNA was applied to 6% polyacrylamide gel with urea and deionized formamide at concentrations from 1.4 M to 4.2 M, and from 8% to 24%, respectively. Electrophoresis was run at 60V and 60°C for 16 h. Gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA), and band presence/absence was manually checked on a blue light transilluminator (Invitrogen, Carlsbad, CA, USA). Similarities of the band profiles were estimated using the Sorensen similarity index (Sørensen, 1957), Cs=2j/(a+b), where j is the number of bands commonly found in both samples, and a and b are the number of bands found in the respective samples compared.

The DGGE bands were excised from the gels using 1 mL pipette tips. The gel pieces were suspended in 100 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid), and the suspensions were frozen and thawed to elute the amplified DNA. The eluted DNA fragments were re-amplified and run on DGGE under the same PCR and electrophoresis conditions. The resultant single bands were excised, recovered, and re-amplified in the reaction volume of 50 μL, and the amplicons were applied onto 1.5% agarose gel electrophoresis at 100V. The bands were excised using a sterilized surgical blade and the amplicons were purified using MonoFas DNA Purification Kit I (GL Science, Tokyo, Japan) with an elution volume of 30 μL. The purified DNA were run on agarose gel electrophoresis and the DNA concentration was checked densitometrically by ImageJ 1.44 (Abramoff et al., 2004).

Thermal cycle nucleotide sequencing of the PCR-amplified 16S rDNA was run using ABI PRISM BigDye Terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems, Carlsbad, CA, USA) with ~5 ng template DNA and a -357F primer without a GC clamp. The products were purified using the BigDye XTerminator Kit (Applied Biosystems, Carlsbad, CA, USA) and analyzed by the ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

PCR products which attained impure sequence results were subjected to cloning. The PCR-amplified DNA fragments were ligated to pT7Blue T-Vector (Merck, Darmstadt, Germany) with the DNA Ligation Kit <Mighty Mix> (Takara Bio, Otsu, Japan), and transformed into *Escherichia coli* DH5α/Competent cells. The cloned DNA fragments were amplified by colony PCR with primers -21M13 Control Primer (Applied Biosystems, Carlsbad, CA, USA) and
BcaBEST Sequencing Primer RV-P (Takara Bio, Otsu, Japan). Nucleotide sequences of the amplified products were determined as shown above.

The closest relatives of the 16S rDNA sequences were determined by the basic local alignment search tool (BLAST) (Altschul et al., 1990). The sequences were also subjected to SeqMatch in the Ribosomal Database Project II (RDP II, Cole et al., 2007), and aligned with the relative 16S rDNA sequences collected from the RDP II database. Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987). The multiple alignment and tree constructions were done with the MEGA version 5 program (Tamura et al., 2011).

Statistical Analysis

Analysis of variance (ANOVA, Steel et al., 1997) was used to estimate the effect of fish feed and magnesium peroxide as well as its concentrations on the variables including moisture contents, pH, redox potential, TOC, TON, total sulfides, and MPN bacterial counts of SRB.

RESULTS AND DISCUSSION

Chemical variables

Redox Potential of the Control sediments (101±3 mV) became highly reduced (-143±3 mV) with the feed input (p<0.05) (Fig.1), suggesting the biodegradation of the loaded organic matter and resultant oxygen consumption by heterotrophic microorganisms. It remained negative (-129±30 mV) in 1% MgO2 but significantly increased to become positive (77±40 mV) in 5% MgO2 (p<0.05), showing that an oxygen-releasing compound (ORC) MgO2 has the potential to improve the reduced sediment conditions. The same trend was observed with total sulfides: the value of the total sulfides significantly increased from 0.79±0.04 mg g⁻¹ to 2.5 mg g⁻¹ with the feed input (Fig. 2), due to the hypoxic conditions under which sulfate reduction starts (Hargrave et al., 2008). This sulfide level is reported to be so critical that benthic animals would disappear (Yokoyama, 2003). Although it stayed high in 1% MgO2 (2.16±0.08 mg g⁻¹), the sulfides significantly decreased in 5% MgO2 (0.37±0.07 mg g⁻¹) which was significantly lower than those in the Control (p<0.05).

The levels of total organic nitrogen (TON) and total organic carbon (TOC) in the treatments were intermediate between the fish farm and non-fish farm areas, according to Kondo (2012), although slight fluctuations were observed. The value of TON significantly increased from 2.71±0.17 mg g⁻¹ to 3.09 mg g⁻¹ (p<0.05) with feed treatment. It did not change with the MgO2 treatments, but a slight decrease to a level similar to that of the control (2.99 mg g⁻¹) was observed in 1% MgO2. There was no significant change in pH, which was at an acceptable level in fish farms (Hargrave et al., 2008), as well as total organic carbon (TOC, 29.04-34.82 mg g⁻¹) and moisture content (64.42-66.01%) (p>0.05) (all of these data are shown in Table 1). It can be said that the chemical variables shown above were not affected by the organic matter load as well as by the ORC addition, at least in our experimental setups, and the microcosm sediments were at a moderately polluted level, regardless of the treatments.

![FIG. 1. Redox potential of the microcosm sediments. Fish feed at 250 g m⁻² was added to the aquaculture farm sediment (Control) and incubated for 20 days. Magnesium peroxide at a concentration of 1% or 5% MgO2 was mixed into the sediment and incubated for 30 more days (1% MgO2, 5% MgO2, respectively). The redox potential was measured with a pH/Eh meter. The means and the standard error of results in triplicate are shown. Superscripts A and B indicate significant differences (p<0.05).](image1)

![FIG. 2. Total sulfides of the microcosm sediments. The experimental setups are the same as described in FIG. 1. The total sulfides were measured with a colorimetric tube detection method. The means and the standard error of results in triplicate are shown. Superscripts A, B, and C indicate significant differences (p<0.05).](image2)
Bacterial community changes

Abundance of sulfate-reducing bacteria (SRB) increased from 3.13 × 10^4 cells g⁻¹ to 12.35 × 10^4 cells g⁻¹ with feed input, decreased to 8.45 × 10^4 cells g⁻¹ in 1% MgO₂, and significantly decreased to 0.24 × 10^4 cells g⁻¹ in 5% MgO₂ (p<0.05) (Fig. 3). This trend is supposed to reflect the sediment conditions: the growth of SRB, stimulated by the reductive environments in the Feed and 1% MgO₂ sediments, was more suppressed under the oxidative conditions in 5% MgO₂ than in the Control.

Changes in the bacterial community were indicated by differences among band profiles of denaturing gradient gel electrophoresis (DGGE) (Fig. 4). The dominance of each bacterial population would change by the ORC addition and resultant oxygen supply, regardless of its concentration, since the Similarity indices of the denaturing gradient gel electrophoresis (DGGE) band profiles of the microbial community in the microcosm sediments. The experimental setups are the same as described in Fig. 1. The total organic nitrogen (TON) and total organic carbon (TOC) were analyzed by a CHNS analyzer. pH was measured with a pH/Eh meter. The moisture contents (Moisture) were measured by a conventional drying-up method. The means and the standard errors of triplicates are shown.

Superscripts A, B, and AB indicate significant differences (p<0.05). Asterisks indicate no significant differences (p>0.05) among the treatments.

TABLE 1. Chemical variables of the microcosm sediments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TON (mg g⁻¹)</th>
<th>TOC* (mg g⁻¹)</th>
<th>pH⁺</th>
<th>Moisture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.71±0.17</td>
<td>29.04±2.60</td>
<td>8.5</td>
<td>64.42±0.22</td>
</tr>
<tr>
<td>Feed</td>
<td>3.09±0.03</td>
<td>30.63±0.23</td>
<td>8.5</td>
<td>64.42±0.22</td>
</tr>
<tr>
<td>1% MgO₂</td>
<td>2.99±0.04</td>
<td>30.10±0.47</td>
<td>8.5</td>
<td>66.01±0.54</td>
</tr>
<tr>
<td>5% MgO₂</td>
<td>3.30±0.03</td>
<td>34.82±1.40</td>
<td>8.5</td>
<td>64.76±1.27</td>
</tr>
</tbody>
</table>

The experimental setups are the same as described in Fig. 1. The total organic nitrogen (TON) and total organic carbon (TOC) were analyzed by a CHNS analyzer. pH was measured with a pH/Eh meter. The moisture contents (Moisture) were measured by a conventional drying-up method. The means and the standard errors of triplicates are shown.

Superscripts A, B, and AB indicate significant differences (p<0.05). Asterisks indicate no significant differences (p>0.05) among the treatments.

**FIG. 4.** Denaturing gradient gel electrophoresis (DGGE) band profiles of the microbial community in the microcosm sediments. The experimental setups are the same as described in Fig. 1. Microbial DNA was extracted from the sediments, 16S ribosomal RNA genes were PCR-amplified, and subjected to DGGE. The arrows with the numbers indicate excised bands which were subjected to nucleotide sequencing and phylogenetic analysis.

**TABLE 2.** Sørensen Similarity Indices of the denaturing gradient gel electrophoresis (DGGE) band profiles obtained from the microcosm sediments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>j</th>
<th>a</th>
<th>b</th>
<th>a+b</th>
<th>Cs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs Feed</td>
<td>10</td>
<td>14</td>
<td>15</td>
<td>29</td>
<td>0.68</td>
</tr>
<tr>
<td>Control vs 1% MgO₂</td>
<td>9</td>
<td>14</td>
<td>17</td>
<td>31</td>
<td>0.58</td>
</tr>
<tr>
<td>Control vs 5% MgO₂</td>
<td>6</td>
<td>14</td>
<td>17</td>
<td>31</td>
<td>0.51</td>
</tr>
<tr>
<td>Feed vs 1% MgO₂</td>
<td>11</td>
<td>15</td>
<td>17</td>
<td>32</td>
<td>0.50</td>
</tr>
<tr>
<td>Feed vs 5% MgO₂</td>
<td>9</td>
<td>15</td>
<td>17</td>
<td>32</td>
<td>0.56</td>
</tr>
<tr>
<td>1% MgO₂ vs 5% MgO₂</td>
<td>12</td>
<td>17</td>
<td>17</td>
<td>34</td>
<td>0.70</td>
</tr>
</tbody>
</table>

The experimental setups are the same as described in Fig. 1. Based on the presence/absence of the DGGE bands, the number of bands common in both samples and unique to either sample were counted and Sørensen Similarity Indices Cs were calculated. The formula is: Index $Cs = 2j / (a+b)$, where j is the number of bands common in samples 1 and 2; a, the number of bands in sample 1; b, the number of bands in sample 2.
### TABLE 3. Presence of the DGGE bands obtained from the microcosm sediments and their most homologous taxa based on BLAST and RDP homology searches

<table>
<thead>
<tr>
<th>Control</th>
<th>Feed</th>
<th>1% MgO</th>
<th>5% MgO</th>
<th>Band No.</th>
<th>Taxonomic groups</th>
<th>Relative species (Accession No.)</th>
<th>Identities (%)</th>
<th>Bacterial characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>1</td>
<td>CFB</td>
<td>99</td>
<td>Aerobe</td>
<td>Nedashkovskaya et al. (2008)</td>
</tr>
<tr>
<td>p</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>2</td>
<td>CFB</td>
<td>Lacinutrix algicola (S000600398)</td>
<td>99</td>
<td>Aerobe</td>
<td>Kim et al. (2008)</td>
</tr>
<tr>
<td>p</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>17</td>
<td>CFB</td>
<td>Cytophaga ferments (S000436362)</td>
<td>91</td>
<td>Facultative anaerobe</td>
<td>Miroshnichenko et al. (2003)</td>
</tr>
<tr>
<td>p</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>20</td>
<td>CFB</td>
<td>Deferrribacter</td>
<td>84</td>
<td>Strict anaerobe, NRB</td>
<td>Fracek and Stolz (1985)</td>
</tr>
<tr>
<td>p</td>
<td>a</td>
<td>p</td>
<td>p</td>
<td>4</td>
<td>CFB</td>
<td>Spirochaetes bivitacilalens (S000324036)</td>
<td>88</td>
<td>Strict anaerobe</td>
<td>Nichols et al. (2005)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>a</td>
<td>a</td>
<td>13</td>
<td>Chlorobi</td>
<td>Prosthecococcus aestuarii (S000290818)</td>
<td>97</td>
<td>Anaerobe</td>
<td>Takashima et al. (2000)</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>3</td>
<td>Chlorobi</td>
<td>Formosa sponcicola (S000199262)</td>
<td>99</td>
<td>Strict anaerobe</td>
<td>Yoon and Oh (2005)</td>
</tr>
<tr>
<td>p</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>7</td>
<td>CFB</td>
<td>Muricauda aquimaria (EU440979)</td>
<td>99</td>
<td>Strict anaerobe</td>
<td>Yoon et al. (2005)</td>
</tr>
<tr>
<td>p</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>6</td>
<td>CFB</td>
<td>Nigrebacter album (AB478415)</td>
<td>84</td>
<td>Strict anaerobe</td>
<td>Iino et al. (2010)</td>
</tr>
<tr>
<td>p</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>15</td>
<td>Fimbicutes</td>
<td>Bacillus cellulosilyticus (NR_040860)</td>
<td>96</td>
<td>Aerobe, NRB</td>
<td>Nogi et al. (2005)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>10</td>
<td>CFB</td>
<td>Bacillus ferments (S000436362)</td>
<td>91</td>
<td>Facultative anaerobe</td>
<td>Bachmann et al. (1995)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>p</td>
<td>p</td>
<td>16</td>
<td>Fimbicutes</td>
<td>Bacillus benzoaevorans (S000130255)</td>
<td>99</td>
<td>Facultative anaerobe</td>
<td>Bachmann et al. (1995)</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
<td>p</td>
<td>p</td>
<td>5</td>
<td>Fimbicutes</td>
<td>Tissierella praeacuta (S000260077)</td>
<td>90</td>
<td>Strict anaerobe, NRB, SRB</td>
<td>Pichinoty et al. (1984)</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
<td>a</td>
<td>p</td>
<td>9</td>
<td>CFB</td>
<td>Mariniabilia salmonicolor (AB080721)</td>
<td>91</td>
<td>Anaerobe</td>
<td>Suzuki et al. (1999)</td>
</tr>
<tr>
<td>a</td>
<td>a</td>
<td>a</td>
<td>p</td>
<td>14</td>
<td>CFB</td>
<td>Alkalifexus imshenetski (S000440489)</td>
<td>90</td>
<td>Anaerobe</td>
<td>Zhilina et al. (2004)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>p</td>
<td>a</td>
<td>19</td>
<td>SRB</td>
<td>Unknown SRB (U08386)</td>
<td>100</td>
<td>SRB</td>
<td>Devereux and Mundfron (1994)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>a</td>
<td>a</td>
<td>8</td>
<td>CFB</td>
<td>Marinifilum fragile (NR_044597)</td>
<td>89</td>
<td>Facultative anaerobe, NRB</td>
<td>Na et al. (2009)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>a</td>
<td>a</td>
<td>11</td>
<td>Chloroflexi</td>
<td>Dehalogenimonas ykanthroporepelliens (NR_044560)</td>
<td>89</td>
<td>Strict anaerobe</td>
<td>Moe et al. (2009)</td>
</tr>
<tr>
<td>a</td>
<td>p</td>
<td>a</td>
<td>a</td>
<td>18</td>
<td>Spirochaetes</td>
<td>Spirochaeta smaragdinae (S000438494)</td>
<td>88</td>
<td>Strict anaerobe, SRB</td>
<td>Magot et al. (1997)</td>
</tr>
</tbody>
</table>

Presence/absence of the bands shown in Fig. 4 is shown as "p" or "a", respectively.

The band numbers are the same as the band numbers shown in Fig. 4.

Most homologous taxa based on the basic local alignment search tool (BLAST) or SeqMatch in the Ribosomal Database Project II (RDP II) are shown with their accessions numbers and identities.

Oxygen requirements as well as potential to reduce sulfate or nitrate of the relative species are shown. SRB, sulfate-reducing bacterium; NRB, nitrate-reducing bacterium.
ces (Cs) estimated from the DGGE band profiles were relatively low between the Feed and 1%/5% MgO. (Table 2). In addition, although the sediment environments were recovered at the same level of the Control, as shown in the redox potential and the total sulfides (Figs 1 and 2), the bacterial community would not return to the former state, based on the low similarity between the Control and 1%/5% MgO. (Fig. 4). A study in Tasmanian salmon farms also showed that a fallow period induced a shift in the bacterial community of the sediments, but dissimilar to the one before the farms had been introduced.
Amplicons of 16S ribosomal RNA genes (16S rDNA), excised from DGGE bands, that are common or unique among the treatments, were sequenced and subjected to homology searches as well as phylogenetic analysis. The results are summarized in Table 3 and Fig. 5, respectively. Among the DGGE bands, bacteria belonging to a *Cytophaga-Flexibacter-Bacteroides* (CFB) group were dominant. The same phenomenon was observed in gilthead seabream cages in Ligurian Sea, the northwest Mediterranean Sea (Vezzulli et al., 2002) and in salmon cages in Tasmania, Australia (Bissett et al., 2006).

SRB closely related to a spirochete *Spirochaeta smaragdinae* (band 18) and the unknown SRB (band 19), which were not found in the control sediment, emerged after the organic matter loading (Feed), and disappeared in the 5% MgO2 sediment. Nitrate-reducing anaerobes, which are relatives of *Marinilimnium fragile* (band 8) and *Bacillus benzocevorans* (band 16), showed the same kinetics as SRB. It reflects a negative redox potential (Fig. 1) and the resultant highly anoxic environment of the Feed sediment and their amendment in 5% MgO2, and was coherent to the viable counts of SRB (Fig. 3).

Four bands related to strictly aerobic bacteria could no longer be found in the anoxic, sulfidic sediment (Feed). Two of these bands were closely related to strict aerobes *Formosa spongiola* (Band 3) and *Muricauda aquimarin* (Band 7). Only one band, a relative of aerobic *Ollela marilimosa* (Band 4), re-appeared with oxygen accumulation by 1% MgO2. This may indicate that most aerobic bacteria will not recover with oxidation after an extreme sulfidic condition. On the other hand, the MgO2 treatment created unique bands, not visible in the Control and Feed.

They include a NRB related to *Marinilabilia salmonicolor* (band 9) and a nitrate- and sulfate-reducing bacterium *Tissierella praecuta* (band 5).

**Factors influencing the bacterial community**

Based on the Sørensen similarity indices and nucleotide sequence identities of the DGGE bands, the bacterial populations were influenced by the addition of fish feed and the ORC, MgO2. Fluctuation in the redox potential and sulfide concentration affects microbial enzymatic activity in the sediments (Hoppe et al., 1990): inhibition of the enzymatic activity was observed in marine fish farms where sulfidic environment dominates (Reichardt et al., 2011). On the other hand, MgO2 addition decreased sulfides inhibiting the enzymatic activity and might bring oxidizing substances such as oxygen and nitrate back into the environment. In contrast, Vezzulli et al. (2004) reported that oxic fish farm sediments did not increase microbial enzymatic activities but enhanced the efficiency of the bacterial carbon utilization in their ORC experiment. They suggested that the ORC potential might be more visible in highly reduced environment.

Table 4 summarizes relationships among the sediment conditions, chemical variables, and bacterial communities based on this study. Addition of fish feed made the sediment condition anoxic with a low redox potential and high total sulfides. Aerobes including *F. spongiola, M. aquimarin*, and *O. marilimosa* disappeared, but the presence of anaerobic *Prosthecocloris aestuarii* was maintained. On the other hand, SRB, which emerged in the anoxic condition, disappeared in the amended sediment, since the redox potential returned to be as high as that in the untreated setup. However, the SRB still persisted in 1% MgO2, due to the transition from anoxic to amended conditions. *Ollela marilimosa*, which was not found in Feed, re-appeared, suggesting its higher

**TABLE 4. Shift in bacterial community in sediments from oxidized to unoxidized, transitory and re-oxidized conditions**

<table>
<thead>
<tr>
<th>Sediment condition</th>
<th>Treatments</th>
<th>Redox potential (mV)</th>
<th>MPN-SRB (×10^4 cells g^-1)</th>
<th>Total sulfides (mg g^-1)</th>
<th>Microbial indicators^a^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxic</td>
<td>None</td>
<td>101</td>
<td>3.13</td>
<td>0.79</td>
<td><em>F. spongiola, M. aquimarin, O. marilimosa, P. aestuarii</em></td>
</tr>
<tr>
<td>Anoxic</td>
<td>Feed</td>
<td>-143</td>
<td>12.35</td>
<td>2.5</td>
<td><em>P. aestuarii, Unknown SRB</em></td>
</tr>
<tr>
<td>Slightly oxidized</td>
<td>1% MgO2</td>
<td>-129</td>
<td>8.45</td>
<td>2.16</td>
<td>Unknown SRB, <em>O. marilimosa</em></td>
</tr>
<tr>
<td>Oxidized</td>
<td>5% MgO2</td>
<td>77</td>
<td>0.24</td>
<td>0.37</td>
<td><em>O. marilimosa</em></td>
</tr>
</tbody>
</table>

^aSpeculated sediment conditions in terms of oxygen contents and redox potential are shown.

^bSpecies, which are the closest relatives of the DGGE band sequences found in the respective treatments and show more than 97% identities, are listed.
tolerance under anaerobic and reduced conditions.

TOC and TON were not key factors in the bacterial community change, since these values were relatively invariant among the treatments. The same phenomenon was observed, in which bio-stimulation through the use of MgO2 intercalated with phosphate was not able to mobilize carbon (Vezzulli et al., 2004).

Environmental application

The need for bioremediation in aquaculture farm vicinities is increasing, as organic matter input creates an anoxic, sulfidic environment that is toxic to the benthic fauna. Bio-stimulation, such as the use of ORC, is a prerequisite in the aquaculture farms where anoxic, sulfidic sediments inhibit enzymatic degradation of protein-rich fish feed waste (Reichardt et al., 2011). The present study proved the applicability of MgO2 in highly reduced aquaculture farm sediment. The bacterial community shift also testified to its effectiveness in improving the sediment conditions. In addition, MgO2 also has favorable characteristics: it releases oxygen slowly but continuously for 4-8 months because of its low oxygen-releasing rate (1.57 × 10^3 mg L^-1 h^-1) compared to the other ORC (Waite et al., 1999). The longevity of this slow-releasing compound gives the edge over CaO2 and H2O2 in the polluted environments (Alvarez and Illman, 2006) such as the aquaculture farm sediment, where continuous oxidation is necessary.

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REFERENCES


