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Laboratory reconstruction and nearby sulfide dynamics of a model benthic boundary layer containing sediment-mixed biofilm

Norihisa ISHIBASHI[1)]*, Hiroo SATOH[2)] and Jota KANDA[1)]

Abstract: Sediment-mixed biofilms of microorganisms embedded within an extracellular polymeric substance were reconstructed using laboratory models of seawater-benthic boundaries, and the sulfide dynamics in the boundary zone were examined. Biofilm-covered sediment was obtained from the field and cultured in a beaker of seawater with a granular formula for fish larvae and powdered foraminiferal limestone. In the standing culture, a floating biofilm formed on the liquid surface, and a microbiota capable of mixed biofilm formation was obtained. The mixed biofilm was cultured in a fluidization-free aerobic state, again with nutritional granules and powdered foraminiferal limestone. This culture formed a sediment-mixed biofilm on the bottom of the beaker. Furthermore, a seawater-benthic boundary model was developed on a hydrated substratum with sediment biofilm, using a nylon mesh as the model bottom layer in the culture system. When the floating biofilm was disrupted and then dispersed on the nylon mesh, detectable biofilms formed on the surface of the model bottom. As nutrients were utilized by the microbes, sulfides accumulated beneath the model bottom. Subsequently, the sulfides passed through the model bottom and became detectable in the seawater just above the sediment-mixed biofilm, where a white-turbid layer formed. As revealed by denaturing gradient gel electrophoresis, the layer contained populations of several bacterial species.

Keywords : *sediment mixed biofilm, seawater-benthic boundary, sulfide cycle, pelagic bacterial layer*

1. Introduction

Sediment-mixed biofilms (SMBs) are aggregations of microbial growth present on the surfaces of most substances in contact with water. They are commonly found on the sedimentary surfaces of seabeds and intertidal zones (BOUDREU and JØRGENSEN, 2001; GHANNOUM and O'TOOLE, 2004). However, the roles of SMBs in the biogeochemical cycle at seawater-benthic boundaries remain unclear. Biofilms have been experimentally grown from individual microbes isolated from natural samples using microtiter plate culture (O'TOOLE and KOLTER, 1998) and flow-chamber culture (WOLFAARDT *et al*., 1994). Mixed biofilms (containing multiple microbial species), such as those occurring on sediments, are more

1) Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4–5–7 Konan, Minato-ku, Tokyo 108–8477, Japan
2) 6–6–9–105 Kameido, Koto-ku, Tokyo 136–0071, Japan
* Corresponding author:
Tel: + 81–80–1301–4588
Fax: + 81–467–24–5026
E-mail: nori2601@yahoo.co.jp

difficult to reproduce in the laboratory. In Japan, fish larvae have been cultured in large, on-land water chambers (with capacities of 10–50 m^3) with powdered foraminiferal limestone as an additive since the early 1990s. Powdered foraminiferal limestone is known to increase the survival of fish larvae (Suzuki *et al*., 2008), but the underlying mechanism is unknown. In 2002, when powdered foraminiferal limestone was present at the Minami-Izu Aquaculture Center (Shizuoka, Japan), the sediment surface was covered by an SMB (Iijima *et al*., 2009). This suggests that SMBs are promoted by powdered foraminiferal limestone, which may be associated with good water quality. Based on these observations, we initiated experimental attempts to reproduce an SMB in a beaker via the addition of foraminiferal limestone.

SMBs can form on two types of substrata in sea beds and intertidal zones: water-containing types (soft bottom or aquatic sediments such as mud and sand, referred to here as "aquatic sediments") and solid types (hard bottom or solid substrata such as rocks, referred to here as "solid substrata"). In aquatic sediments, solid substances such as sand and mud are mixed with organic matter and infused with pore water (Boudreu, 1997). In the seawater-benthic boundary zones at the bottom of shallow sea areas, abundant organic matter sedimentation accumulates from both land and the aquatic environment and is utilized and degraded by many species of microbes (Koike, 2000; Fenchel *et al*., 2012). Owing to the vigorous degradation of organic matter, remarkable oxygen losses occur at the benthic boundary, and the high concentrations of sulfate ions in the anaerobic marine environment activate microbial sulfur metabolism. Consequently, a great variety of sulfur compounds is formed (Gemerden, 1993; Samukawa and Hiiro, 1996; Rickard, 2012). SMBs have been widely investigated on solid substrata (Riding and Awramike, 2000), but their roles in aquatic sediments are less well understood. The laboratory study of the sulfur cycles in a sandy beach habitat using an experimental chamber suggested that bacterial biofilms on sand substrata produce much of the sulfur at the benthic boundaries between seawater and sand substrata (Jørgensen, 1974). The sandy beach study indicates that biogeochemical studies of aquatic sediments would be greatly facilitated by the availability of an appropriate laboratory model of aquatic sediments with SMBs.

In the present study, we developed a model experimental system that reconstructs SMBs from naturally occurring microbial assemblages in sediments. We first reproduced SMBs observed on solid substrata in aquaculture tanks and then, using the same method, produced SMBs from natural sediments with bacterial assemblages collected at a variety of sites in coastal environments. We also established an experimental setup that can model an aquatic sediment in the laboratory and produced SMBs on the surface of the modeled aquatic sediment. In order to demonstrate that the experimental model could be used in biogeochemical studies of SMBs, we then analyzed the water-quality parameters (sulfide levels and dissolved oxygen) and bacterial species at the benthic boundary of the model. Experiments were performed in the laboratory of the Minami-Izu Aquaculture Center from 2002 to 2003 and at the Tokyo University of Marine Science and Technology (Tokyo, Japan) from 2009 to 2016. While the laboratory SMBs we obtained may not completely reproduce or be representative of the natural SMBs in coastal environments, further improvement of the reproduction technique will hopefully result in the establishment of a conventional laboratory model for SMBs in natural coastal environments.

Table 1. Composition of "Ambrose 400" nutrition granules.

Component	Content (w/w%)
Crude protein	≥ 52
Crude fat	≥ 8.0
Crude fiber	≤ 3.0
Crude ash	≤ 15
Calcium	≥ 2.0
Phosphorus	≥ 1.4

SOURCE: NIPPON FORMULA FEED MANUFACTURING (2009)

Table 2. Mineral element composition of "Fish Green" foraminiferal limestone powder.

Mineral element	Content (g/kg)
Calcium (Ca)	302
Silicon (Si)	71.7
Magnesium (Mg)	5.94
Aluminum (Al)	5.9
Iron (Fe)	4.62
Sodium (Na)	2.7
Potassium (K)	0.74
Phosphorus (P)	0.21
Manganese (Mn)	0.2
Zinc (Zn)	0.4

SORCE: GREEN CULTURE (2000)

2. Materials and Methods

To reproduce the SMBs of an aquaculture facility, we collected SMB-containing sediments with a spatula from the bottom of a concrete water chamber (capacity, 20 m^3) in which fish larvae were reared at the Minami-Izu Aquaculture Center of the Japan Sea-farming Association (currently the Minami-Izu Aquaculture Center of the Fisheries Research and Education Agency, Minami-Izu, Shizuoka, Japan; 34° 36′43″ N, 138° 50′55″ E). Moreover, seabed sediments containing SMBs were collected from three marine sites during the spring ebb tide at a water depth of about 0.1 m. For sampling, an open, transparent acrylic cylinder (length, 20 cm; inside diameter, 3 cm) was thrust into the benthic layer from above and then immediately closed at both ends. The sites included a canal under the TENNOUZU Bridge (Minato-ku, Tokyo, Japan; 35° 37′24″ N, 139° 44′42″ E), a canal frontage site at OHI Seaside Park (Ota-ku, Tokyo, Japan; 35° 35′ 19″ N, 139° 44′58″ E), and a shoreline off the SHICHIRIGAHAMA sand beach (Kamakura, Kanagawa, Japan; 35° 18′9″ N, 139° 31′26″ E). The two former sites occupy the innermost part of Tokyo Bay, and the latter is located along Sagami Bay. Surface substratum samples were also collected from an offshore seabed near HANEDA in Tokyo Bay (35° 30′42″ N, 139° 49′48″ E) at an approximate depth of 30 m using an Ekman-Birge bottom sampler (RIGO, Tokyo, Japan). Each substratum sample was submerged in seawater and transported to the laboratory.

The formation of floating biofilms was investigated in standing cultures of microbes nourished with a granular fish-larvae feed [Ambrose 400; Nippon Formula Feed Manufacturing (currently, Feed One), Yokohama, Kanagawa, Japan; particle diameter, 420–650 μm] used as a complex nutrient for microbes, hereafter referred to as nutritional granules, and powdered foraminiferal limestone as an additive (Fish Green, Green Culture, Takaoka, Toyama, Japan; average diameter, 70 μm). The constituents of the nutritional granules and foraminiferal limestone powder are listed in Tables 1 and 2, respectively. For standing culture, the SMB-containing sediment, powdered foraminiferal limestone, and nutritional granules were spread over the bottom of a 100–cm^3 glass beaker or a 220–cm^3 plastic vessel with 80 cm^3 of sand-filtered seawater, which was covered with PVDC film (Kureha, Tokyo, Japan) to prevent evaporation.

Figure 1 shows the culture model system of

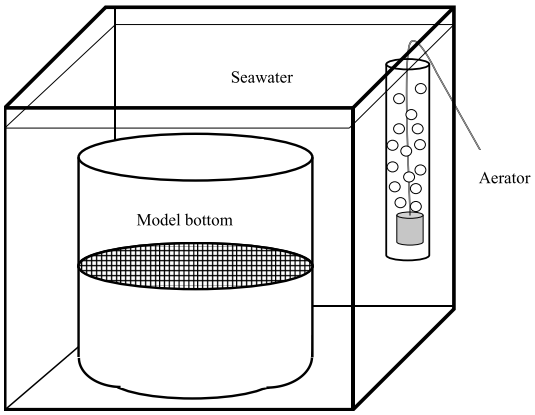


Fig. 1. Experimental model of biofilm formation at the aquatic benthic boundary. The model bottom (nylon mesh, 50 μm) was affixed in a transparent acrylic vessel (diameter, 9.5 cm; height, 15 cm) with a closed bottom and an open mouth. The acrylic vessel was submerged in a glass chamber of dimensions 18 cm (D) × 30 cm (W) × 24 cm (H).

the seawater-benthic boundary with the sediment biofilm on hydrated substrata. To develop the culture model system, we placed a nylon mesh (pore size, 50 μm; NYTAL DIN1 10–50, Sefer, Ruschlikon, Switzerland) at the mid-height of a transparent cylindrical vessel (acrylic; inside diameter, 9.5 cm; height, 15 cm) with an open upper end. This system models the bottom surface of a typical aquatic sediment. The use of nylon limits the disintegration that might occur under microbial chemical activity. The space below the mesh was filled with seawater, modeling the pore water of aquatic sediments. This vessel was placed in a compact, glass water chamber (area, 18 cm × 31 cm; height, 24 cm). The seawater in the water chamber was maintained in a fluidization-free aerobic state. At the Minami-Izu Aquaculture Center, where an SMB covers the bottom of the aquaculture tank, the bottom layer of water is static because the air stones are suspended at an intermediate depth in the rearing chamber. However, the oxygen saturation in this layer is ≥ 70%. To reproduce these conditions in the bottom-layer water in the aquaculture tank, we installed an air stone (diameter, 3 cm) in a plastic cylinder (length, 12 cm; diameter, 5 cm) with numerous 3–mm-diameter pores to prevent water movement of the culture system. The system was aerated with an air pump (O3PROOF202, Addx, Hachioji, Japan). Test cultures were prepared with sand-filtered and mat-filtered seawater. The filter mat was a filtering material for aquarium water (Mat Kobo, Tokyo, Japan). The salinity of the filtered seawater was not below 15.0, and the indoor light intensity of the laboratory was not higher than 6.8 $\mu E \cdot m^{-2} \cdot s^{-1}$. Culture experiments were initiated by spreading the seed bacterial population, powdered foraminiferal limestone, and nutritional granules on the nylon surface.

To quantify biofilm formation, we measured the packed cell volume (PCV) and the wet weight. Biofilm samples were placed in a graduated 10–cm^3 tube and centrifuged (H107, Kokusan Centrifuge, Tokyo, Japan) at 2000 rpm for 3 min, and the volume was defined as the PCV. To determine the wet weight of each biofilm, the floating biofilm on the liquid surface was transferred to a nylon mesh (5 × 5 cm; pore size 50 μm; NYTAL DIN1 10–50, Sefer, Ruschlikon, Switzerland), drained of the biofilm surface water by applying water-absorbing paper from below the mesh, and weighed repeatedly on a precision balance until the weight was nearly constant. The biofilm was observed both macroscopically and microscopically (CK2, Olympus, Tokyo, Japan).

Total acid volatile sulfides (AVSs) were measured using the detector tube method (HEDRO-TECH–S330, Gastec, Ayase, Japan). AVSs are sulfide compounds that volatilize in water under

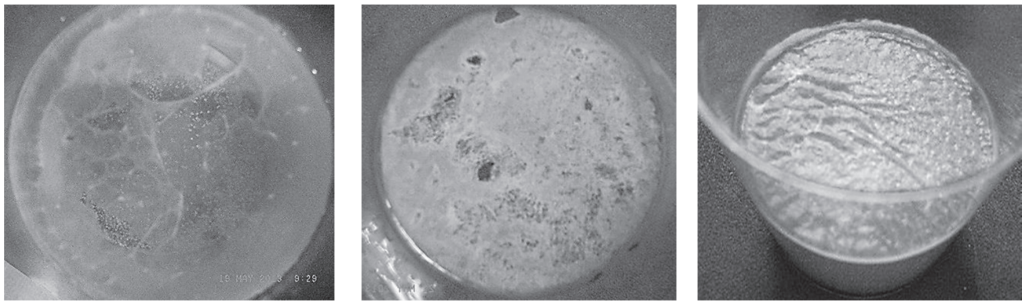
acidic conditions, namely, hydrogen sulfide, hydrogen sulfide ions, sulfide ions, and sulfur from non-pyrite iron sulfide. The dominant species are H_2S and HS^- (DAVID and MORSE, 2005; SUGAHARA *et al*., 2012). Hereafter, the total AVSs measured using this method are simply referred to as sulfides or AVSs. The bacterial density or turbidity of the culture water was assayed by determining the absorbance at 660 nm (OD_{660}) in a spectrophotometer (UV160, Shimadzu, Kyoto, Japan; MARDIGAN *et al*., 2009). The dissolved oxygen (DO) content was measured using a DO meter (YSI-55, Yellow Spring Instrument, Yellow Spring, OH, USA). For the culture system modeling the seawater-benthic boundary of an aquatic sediment, the sensor of the DO meter was vertically inserted at a non-water-agitating speed (4 cm/min) using a slow elevator (made in-house). The water samples from above the model bottom surface that were used for measuring sulfide concentrations and turbidity were collected with a syringe (5 cm^3) joined to a silicon tube (inside diameter, 2.0 mm) fixed to the side of the sensor. The water was sampled at a depth of approximately 2 cm using another syringe (capacity, 1 cm^3) and a syringe needle to penetrate the mesh. Oxidation-reduction potential (ORP) was measured using an ORP meter (RM-12P, DKK-TOA, Tokyo, Japan).

The microbial composition in the white-turbid layer was analyzed by denaturing gradient gel electrophoresis (DGGE) (MUYZER and SMALLA, 1998; ISHII *et al*., 2000; NISHIJIMA *et al*., 2010). Samples were frozen at −80 ℃ until required for DGGE analysis. DNA was extracted using MORA-EXTRACT (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), and the bacterial 16S rDNA was amplified by a modified touchdown polymerase chain reaction (PCR) method (DON *et al*., 1991; MUYZER and SMALLA, 1998). A 16S rDNA sequence of approximately 200 bp was amplified using the 341f-GC and 534r primers. Electrophoresis was performed at 100 V for approximately 12 h. The electrophoresis mixture included the PCR product, 8% (w/v) polyacrylamide gel, and denaturant at concentrations of 25–65%. To check the DNA purity over the band width, the DNA from a band in the electrophoresed gel was extracted and used as the template in a second PCR. The second amplification product was labeled using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), and its nucleotide sequence was determined by an ABI 3130xl Genetic Analyzer System (Applied Biosystems). A homology search and simplified molecular phylogenetic analysis were performed using the Apollon 2.0 program (Techno Suruga Laboratory, Shizuoka, Japan), the International Base Sequence Database (Gen Bank; DDBJ; EMBL), and the Apollon Reference Strain Database DB-BA9.0 (Techno Suruga Laboratory).

3. Results

3.1 Reconstitution of sediment-mixed biofilms above the model bottom surface

In the standing culture of SMB-containing sediment collected from the bottom surface of the water chamber used for rearing fish larvae (sediment weight/base area, about 100 mg/cm^2), containing added powdered foraminiferal limestone (5 mg/cm^2) and nutritional granules (10 mg/cm^2), a floating membrane containing bacteria formed on the surface of the liquid on day 4 of cultivation at 22 ℃. Similarly, cultivation with natural coastal sediments collected from the aforementioned sites also yielded floating membranes. With nutritional granules of varying areal densities (5, 10, 15, or 20 mg/cm^2) and powdered foraminiferal limestone (3 mg/cm^2), we examined in detail the formation of the floating biofilm produced from the substratum col-



(a) (b) (c)

Fig. 2. Formation of floating mixed biofilm by the aquatic standing-culture method (20 ℃). (a) Liquid sol-like biofilm (day 4 of cultivation). (b) Solid gel-like biofilm (day 7 of cultivation). (c) Gel-like colloidal biofilm deprived of surface water (day 9 of cultivation).

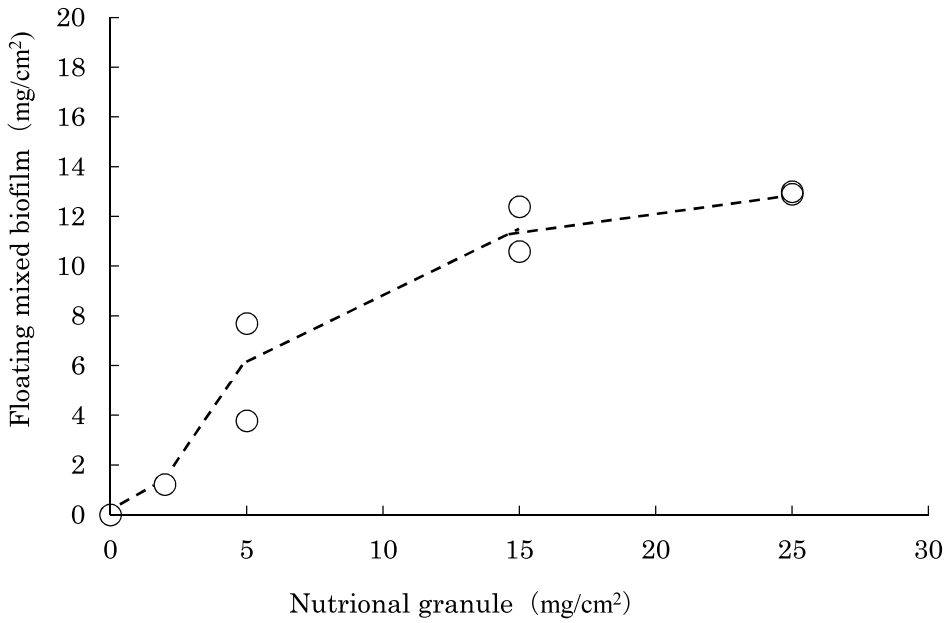


Fig. 3. Formation of floating mixed biofilms nourished with different amounts of nutritional granules. The weights of the floating biofilms after 7 days of culture at 20 ℃ are shown.

lected from a canal under the TENNOWZE Bridge (100 mg/cm^2). On day 4, a highly fluid, sol-like membrane formed on the liquid surface (Fig. 2 (a)). On day 7, the membrane became thick, elastic, gel-like, and less fluid (0.6 cm^3 PCV with both the 10 mg/cm^2 and 15 mg/cm^2 nutritional granules; Fig. 2 (b)). On day 9, the gel-like membrane formed using the 20 mg/cm^2 nutritional granules became surface-water depleted and fragmented (Fig. 2 (c)). In contrast, those formed using 5, 10, or 15 mg/cm^2 nutritional granules maintained their gel-like properties with a wet surface. Figure 3 shows the relationship between biofilm formation and nutrient lev-

el. The wet weight of the biofilm increased as the areal density of the nutritional granules increased from 3 to 15 mg/cm^2. However, increasing the areal density of the nutritional granules to 25 mg/cm^2 yielded no further increase in biofilm growth.

In the culture experiments with a 100-cm^3 beaker under fluidization-free aerobic conditions, where the culture contained a mixture of biofilm-containing sediment from the fish-larvae culture tank (weight/base area, 100 mg/cm^2), nutritional granules (10 mg/cm^2), and powdered foraminiferal limestone (5 mg/cm^2), the water above the sediments in the beaker became transparent, and an SMB formed on the sediment surface on day 4 of cultivation at a water temperature of 22 ℃. Under the same conditions, we cultured the floating biofilms (0.2 cm^3 PCV) obtained from the cultivation of the sediment-attached biofilms collected from the four marine locations. The resulting SMBs were similar in appearance and developmental processes to those formed from the sediment collected from the aquaculture tank, indicating that SMBs can be reconstructed on a solid substratum using the culture conditions adopted in this study.

3.2 Sulfide and microbial dynamics near the aquatic benthic boundary of the SMB model

The above-described procedure was applied to a model aquatic sediment. To create the model aquatic sediment (Fig. 1), the floating biofilm (0.2 cm^3 PCV) grown from sediments collected under the TENNOUZ Bridge was disrupted and spread over the nylon bottom surface as the seed microbial population, together with nutritional granules (10 mg/cm^2) and powdered foraminiferal limestone (3 mg/cm^2). On day 1 of cultivation, the water contained a light suspension within 3 cm above and below the model bottom surface. After 2 days, the slightly turbid

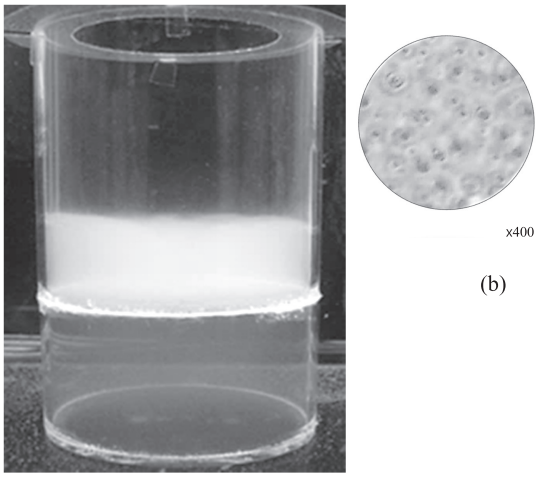


Fig. 4. White-turbid layer above the bottom of the modeled aquatic benthic boundary with biofilm growth after 5 days of culture at 20 ℃ (a), and a microscopic image of bacteria in the white-turbid layer (b).

phase was distinctly separated from the transparent phase of the water. The boundary was observed just above the model bottom surface, on which a biofilm, presumably an SMB, had formed. On day 3 of cultivation, a dense white-turbid layer was formed above the SMB on the model bottom, remaining until day 11 (Fig. 4(a)). This white-turbid layer was confirmed to be contain densely populated microbes under light microscopy. The microbial cells (approximately 3 μm in diameter) contained green granules and were surrounded by a 30-μm thick coat of translucent colloidal material (Fig. 4 (b)). Once this layer had been established, the DO rapidly decreased below the upper surface of the layer. The DO level in the white-turbid layer was close to zero ($\leq$ 0.005 mg/dm^3; Fig. 5). The sulfide concentration was nearly uniform (about 0.2 mg-S/cm^3) in the white-turbid layer above the model bottom surface, slightly lower in the water be-

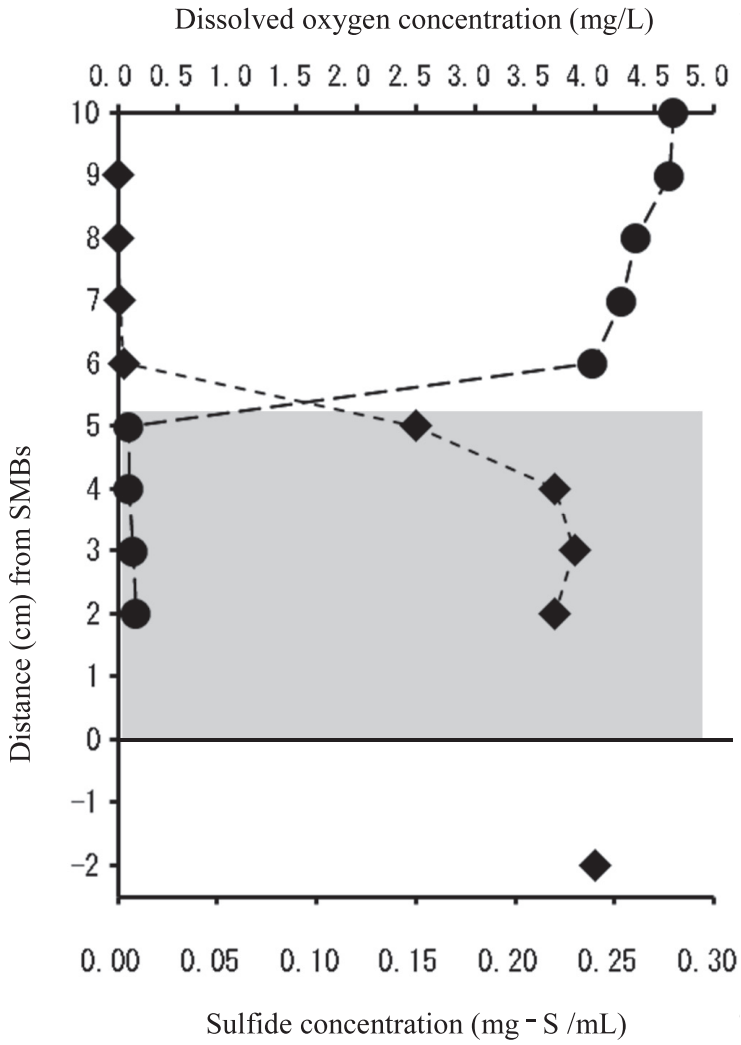
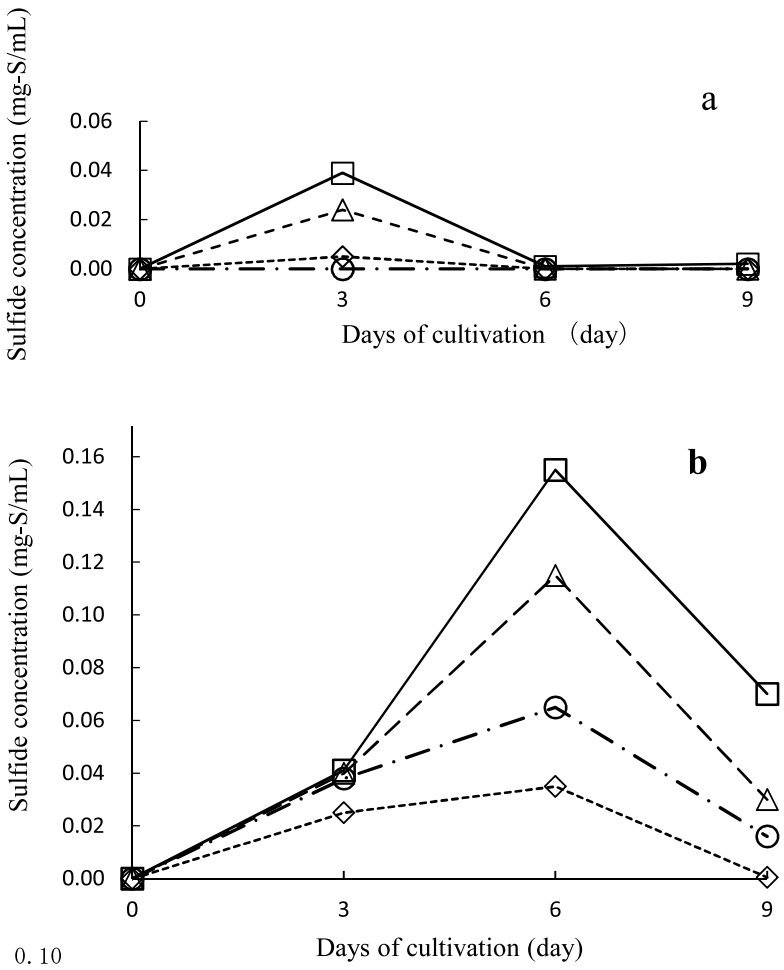


Fig. 5. Vertical distributions of sulfides (AVSs) and dissolved oxygen in the presence of the white-turbid layer in the experimental system after 5 days of culture at 20 ℃. Diamonds, sulfides (AVS); circles, dissolved oxygen (DO); shaded area: white turbid layer.

low the model bottom surface, rapidly decreased above the white-turbid layer, and was undetectable in the transparent water above the white-turbid layer (Fig. 5). The turbidity (OD_{660}) in the white-turbid layer remained essentially unchanged. Above the white-turbid layer, steep gradients (i.e., chemoclines) of DO and sulfide concentrations were observed (Fig. 5). Formations of white-turbid layers with similar patterns of sulfides and DO distribution were observed for the substrata collected from all canal and shoreline sites.

Figure 6 shows the effect of the areal density of the nutritional granules on sulfide formation. Increased amounts of nutritional granules increased the maximum sulfide concentrations in

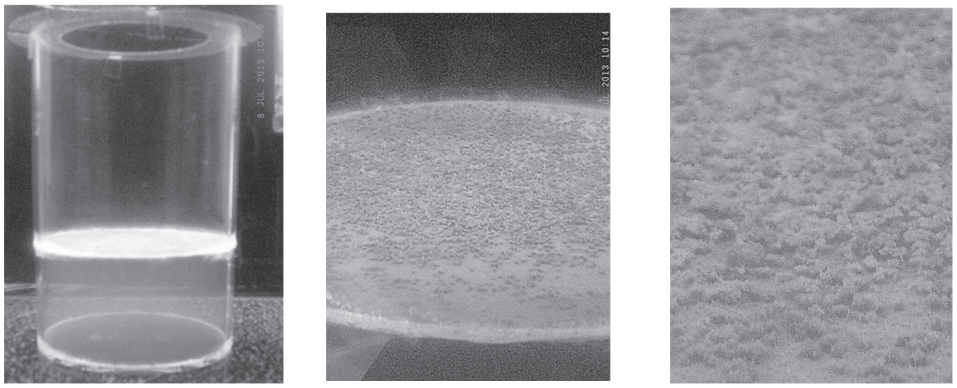


0. 10

Fig. 6. Sulfide concentration in the water 2 cm above (a) and 2 cm below (b) the bottom of the aquatic benthic boundary model (culture water temperature, 25 ℃) with biofilm growth using different amounts of nutritional granules (diamonds, 5 mg/cm^2; triangles, 7.5 mg/cm^2; squares, 10 mg/cm^2; circles, 20 mg/cm^2) with a fixed volume of crushed colloidal biofilm (0.2 cm^3 PCV) and a constant density of powdered foraminiferal lime stone (3 mg/cm^2).

the overlying and underlying water in the models containing 5, 7.5, and 10 mg/cm^2 nutritional granules, as well as prolonging the time to formation of the white-turbid layer just above the model surface. The white-turbid layer was maintained while sulfide was detected in the underlying water. In contrast, in the model containing 20 mg/cm^2 nutritional granules, sulfides (AVSs) in the underlying water were detected on day 3 and peaked on day 6 but were never detected in the overlying water (Fig. 6). In addition, no white-turbid layer formed just above the model bottom surface, and the SMB that formed on the model bottom surface was white and only slight-

(a)



(b)

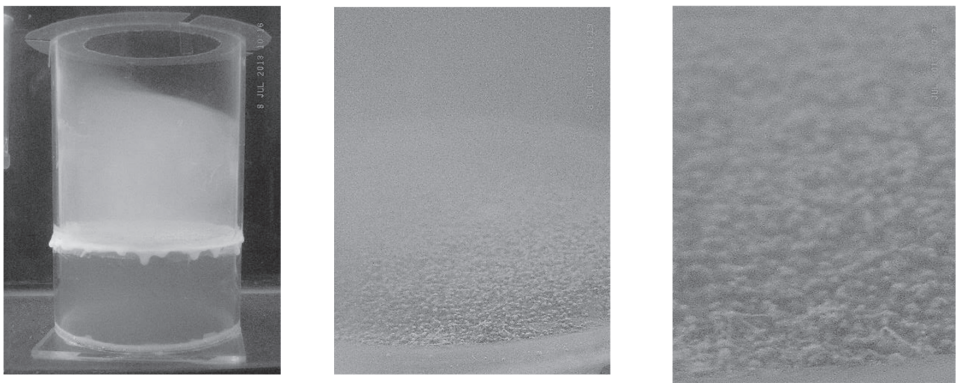


Fig. 7. Absence of white-turbid layer above the model aquatic sediment after 7 days of culture with 20 mg/cm^2 of nutritional granule (a), and the biofilm and the white-turbid layer above the model aquatic sediment after 7 days of culture with 10 mg/cm^2 of nutritional granule (b).

ly viscous (Fig. 7).

Figure 8 shows the effect of temperature on sulfide formation in an experiment with the TENNOUZU sediment. Below the model bottom surface, the maximum sulfide concentration was nearly independent of water temperature, but above the model bottom surface, it increased with decreasing water temperature. The time required to detect the sulfides also increased with decreasing water temperature, both above and below the model bottom surface (Fig. 8). The persistence time of the white-turbid layer increased with decreasing water temperature. Above the model bottom, the ORP of the water rapidly decreased over time; moreover, the ORP reduction rate increased with increasing water temperature. At all of the examined water temperatures, the ORP reached −300 mV on days 3–5. Additionally, the white-turbid layer formed when the ORP reached ≤ −100 mV above the

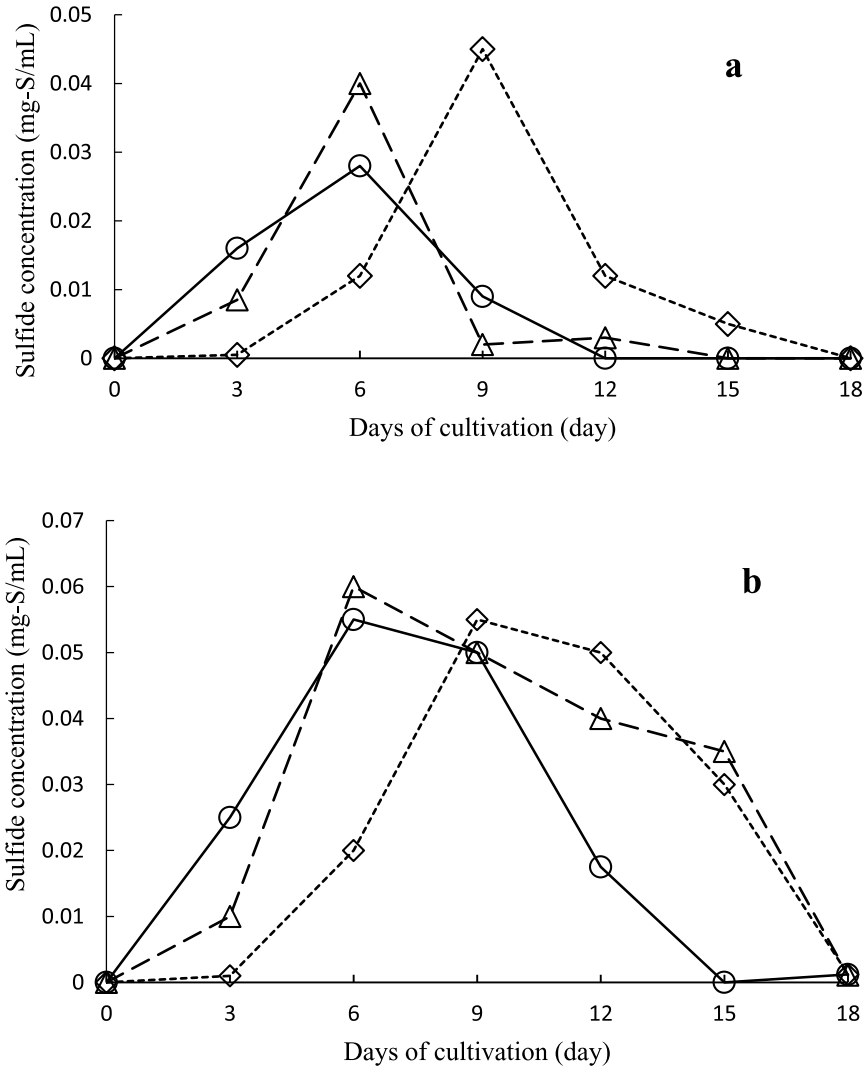


Fig. 8. Sulfide concentration in the water above (a) and below (b) the bottom of the aquatic benthic boundary model (nutritional granules, 5 mg/cm^2) with biofilm growth at different water temperatures. Circles, 25 ℃; triangles, 20 ℃; squares, 15 ℃. The original sediment was collected under the TENNOZU Bridge.

model surface and disappeared when the ORP was restored to ≥ 0 mV above the model surface.

Figure 9 shows the PCR-DGGE results of an experiment with the TENNOUZU sediment indicating the temporal changes in the bacterial community of samples collected from the white-turbid layer above the model bottom surface. Three distinct bands (a, b, and c; Fig. 9) appeared on days 1 and 2, and four distinct bands (a, b, c, and d; Fig. 9) were present on day 3 of cultivation. In the sample on day 3 when the white-turbid layer formed, the intensity of band a was markedly more intense than those of the other two bands (b and c). Sequence analysis

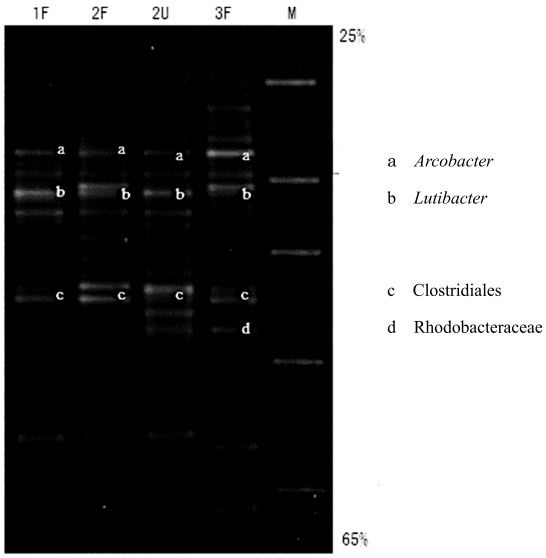


Fig. 9. DGGE electrophoretogram showing the microbial composition of the water above the model bottom from day 1 to day 3 of cultivation (until formation of the white-turbid layer). Lanes 1F, 2F, and 3F were extracted from the water 2 cm above the model bottom on days 1, 2, and 3, respectively. Lane 2U was extracted from the water 5 cm above the model bottom on day 2. Lane M is a DGGE marker (Nippon Reiji, Tokyo, Japan). The culture was created with disrupted biofilm (0.2 cm^3 PCV), nutritional granules (10 mg/cm^2), and powdered foraminiferal limestone (3 mg/cm^2) at 25℃. The original sediment was collected under the TENNOZU Bridge.

indicated that band a was derived from *Arcobacter* sp. (sequence identity, 100%), a microaerophilic sulfur bacterium. Band b was derived from *Lutibacter* sp., with 99% sequence identity to *Lutibacter maritimus*, a marine bacterium of the family Flavobacteriaceae isolated from a tidal flat sediment (CHOI and CHO, 2006), whereas band c was derived from a bacterium of the order Clostridiales (sequence identity, 94%), an obligate anaerobic sulfur-reducing bacterium commonly found in soil. Band d was derived from a bacterium in the family Rhodobacteraceae (sequence identity, 99%), a purple nonsulfur photosynthetic bacterium that utilizes S^0.

4. Discussion

4.1 Reconstitution of SMBs on the model bottom surface

In this study, floating biofilms from sediment microorganisms were formed on liquid surfaces in standing cultures. It has long been known that *Bacillus subtilis* forms a floating microbial film at the air-liquid interface of a standing culture (MORIKAWA *et al.*, 2006; KOBAYASHI, 2007). In the floating biofilm development of *Shewanella oneidensis*, the pellicle forms in three steps, including rapid formation of a thin pellicle, followed by evolution into a heterogeneous biofilm and finally into a thick homogeneous biofilm (ARMITANO *et al.*, 2013). The floating biofilms in our study also showed three developmental stages: a highly fluid, sol-like membrane stage; a thick, elastic, gel-like membrane stage; and a gel-like membrane with a water-depleted surface stage, which possessed a dry, gel-like consistency (Fig. 2). The growth of the floating biofilms in our experiments was therefore similar to that of other known biofilms (LEWANDOWSKI and BEYENAL, 2013).

As suggested by the PCR-DGGE analysis of the microbial assemblage developed using the floating biofilm as the seed population, mixed species of bacteria constituted the floating biofilms. Unlike separation methods involving homogenization or sonication, in which some portions of a microbial assemblage might be destroyed (REIL, 1994), the floating biofilm of this study developed from the natural sediment community under relatively mild conditions. Using this mild separation technique, we were able to produce a mixed biofilm containing a coexisting set of bacterial species.

Our results further showed that, using a floating biofilm as the seed population, SMBs could be cultured on solid substrata and aquatic sediments with nutritional granules and powdered foraminiferal limestone. Among the SMBs obtained in this study, we speculate that the biofilm produced from the sediments of the aquaculture tank should likely reflect the biofilm that was originally found in the aquaculture facility, as the reproduction conditions were essentially the same as those of the aquaculture tank. Studies using this laboratory-grown biofilm would provide valuable information that could lead to the improvement of aquaculture conditions. Using the same technique, we successfully produced mixed biofilms from seed populations obtained from natural coastal sediments with SMBs. Because the growth of each species in a natural bacterial assemblage changes substantially during artificial cultivation, the species composition in the mixed biofilm produced in the laboratory may be different from the seed population. Nevertheless, the successful laboratory development of a mixed biofilm enabled us to examine the possible role of a mixed biofilm in biogeochemical cycles in a laboratory setup. While the mixed biofilm and the material dynamics in the laboratory setup may not completely represent the processes occurring in natural benthic boundary systems, laboratory studies using this system could provide at least preliminary insights. Furthermore, with additional examination and improvement of the conditions used for biofilm formation in this study, it may be possible to develop a laboratory system of SMBs that could be used in biogeochemical studies of natural marine environments.

4.2 Sulfide and microbial dynamics near the aquatic benthic boundary of the SMB model

Laboratory-grown culture systems that can model common aquatic sediments in coastal environments such as sand and mud should contribute to studies of sulfur cycles. JØRGENSEN (1974) conducted an extensive study of sulfur cycles in a sandy beach environment in a water chamber by placing sand in a chamber with seawater and adding seagrass to model the conditions on a post-storm sandy beach. This was used to examine a radioactive isotope of sulfur, with sulfides accumulated below the substratum surface. Approximately 90% of the sulfides migrated and diffused upward from substratum surface. At the end of the experiment, the surface of the seagrass was covered with microbial biofilms, including those of sulfur-reducing bacteria. These findings suggested that mixed biofilms are responsible for sulfide accumulation below the sand surface (JØRGENSEN, 1974). A similar sulfur cycling process was observed in our present study. We found that the permeability of the biofilms depended on the areal density of the nutritional granules (Fig. 6); with an abundance ($20\ mg/cm^2$) of nutritional granules, no sulfide migration from below the substratum surface was observed. It has been generally observed that the biofilm structure is sparse under low-nutrient conditions and dense under high-nutrient conditions (WIMPENNY and COLASSNTI, 1997). This suggests that under abundant nutrient levels, the biofilm structure becomes very dense and blocks sulfide migration.

Unlike the sandy-beach model of JØRGENSEN (1974), our culture model system of the seawater-benthic boundary was fluidization-free above the bottom surface. Therefore, in our study, the sulfides rose from below the substratum surface and accumulated in the water above the model bottom. A dense white-turbid layer formed in the same region. Using a radioactive sulfide, MATSUYAMA (1978) showed that sulfide was formed by sulfur-reducing bacteria in strati-

fied meromictic lakes. In these lakes, the deeper seawater layer is covered by a superficial freshwater layer. However, sulfide formed not in the bottom layers of the water but on the bottom surface, with sulfide concentrations peaking immediately below the bottom surface. Subsequently, the sulfides passed through the bottom surface and accumulated in the anaerobic water immediately above the surface. This sulfur cycle in the benthic boundary of the stratified water of meromictic lakes is generally consistent with the sulfur cycle in our model experiment. Sulfide production and accumulation in bottom-layer water was also observed in the so-called "blue tide" in Tokyo Bay (MARUMO and YOKOTA, 2012). MAKI *et al.* (2013) revealed that sulfides exist in and just above the substratum in the innermost part of Tokyo Bay during the summer. In this part of the bay, the anoxic layer is known to correspond with the sulfide-containing layer just above the bottom (OKADA *et al.*, 2011). The distributions of DO and sulfides in the white-turbid layer just above the model bottom surface in this study were similar to those in Tokyo Bay. Similar white-turbid layers (indicating a pelagic bacterial layer) in the anaerobic water were also observed in previous studies. In the stratified water of the Black Sea and in meromictic lakes such as Lake SUIGETSU and Lake KAIIKE, pelagic bacterial layers consisting mainly of photosynthetic bacteria and sulfur-containing bacteria have been observed at intermediate depths (CANFIELD *et al.*, 2005). Furthermore, the substratum surfaces of Lake Suigets and Lake Kaiike are lined with bacterial mats (MATSUYAMA and SAIJO, 1971; OGURI *et al.*, 2002). KOIZUMI *et al.* (2005) diluted the microbial mat on the bottom surface of Lake KAIIKE and found that mesophilic sulfate-reducing bacteria belonging to the Deltaproteobacteria and Epsilon-proteobacteria play an important role in sulfur metabolism on the sediment surface. The sulfur cycle in our laboratory model thus exhibited several similarities to the sulfur cycles in these natural systems.

In the present study, mixed-species biofilms were obtained in the laboratory by simultaneously separating the species from the substratum. The coexistence of these microorganisms indicates that individual microbes cooperate with each other to maintain biofilm function. Moreover, biofilms are associated with the formation of pelagic bacterial layers just above the bottom surface.

5. Conclusions

SMBs were reconstructed in laboratory models of seawater-benthic boundaries, and the sulfide dynamics in the boundary zone were examined. Floating biofilms were formed on the liquid surfaces of vessels containing seawater, natural sediment samples, nutritional granules, and powdered foraminiferal limestone. The mixed microbial communities of these biofilms can be used as seed populations for reconstructing benthic biofilms on both solid substrata and aquatic sediments in laboratory models. In our benthic boundary model of biofilms and hydrated substrata, anaerobic layers formed in the seawater below and above the aquatic bottom surface. A mixed biofilm then developed on the model bottom surface. The bacteria in this biofilm produced and accumulated sulfides in the seawater below the bottom surface. These sulfides eventually migrated to the anaerobic seawater above the bottom surface and were probably utilized by the pelagic bacterial layer. The results obtained in our model of benthic boundaries suggest that the model exhibits similarities to the natural benthic boundary environments of seawater.

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