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Isolation of heterotrophic thiosulfate-oxidizing bacteria and their role in a conserved tidal flat in the Ariake Sea, Japan

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Intolerable sulfide emission was spotted at several areas in tidal flats of the Ariake Sea, Japan. Sulfide is naturally produced in tidal flats and rapidly oxidized by sulfur-oxidizing bacteria (SOB). This makes them important players in controlling released sulfide by sulfate-reducing bacteria. A part of SOB can grow heterotrophically and we isolated them from a conserved muddy tidal flat in the Midorikawa Estuary, Kumamoto. The obtained heterotrophs oxidized sulfur compounds in presence of organic carbon. Various metabolic pathways were detected among them during oxidation of thiosulfate and an isolate showed capability of sulfide oxidation. Phylogenetically, they were close-related to the genera *Paracoccus*, *Bacillus*, *Dyella*, and *Pseudomonas*. This suggested that the isolated SOB were affiliated to diverse classes and functioned diversely in oxidative side of the sulfur cycle. Additionally, population number of heterotrophic SOB was detected in high abundance, suggesting that they played a significant role in the sulfur cycle of the Midorikawa Tidal Flat.

Key words: Heterotrophic sulfur-oxidizing bacteria, isolation, tidal flat, thiosulfate, sulfide.

INTRODUCTION

Ariake sea is a semi-closed sea that has limited contact to the open ocean for exchange of materials and thereby is susceptible to environmental changes. In ecological term, the sea is dependent on the surrounding tidal flats to control nutrition that flow to the semi-closed sea. However, environmental deterioration of the tidal flats has been observed in recent decades (Du et al., 2008). Substantial decreases in populations of existing benthic animals due to sulfide emission have been reported (Moqsud et al., 2006). Carrion of death animals augmented the amount of existing organic matter, which caused extensive anoxia in the sediments. This worsened the condition as poisonous sulfide was more produced to massive quantities (Du et al., 2008).

Sulfide is normally emitted from the anoxic part of tidal flats near the surface layer of muddy sediments. The compound is excreted by sulfate-reducing bacteria (SRB) as a metabolic waste product from the anaerobic respiration of organic compounds with sulfate that is supplied by seawater during high tide (Muyzer and Stams, 2008). Large amount of sulfide is produced in the sediments, but only insignificant amounts are released to the atmosphere

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(Bodenbender et al., 1999). Small amount of the sulfide reacts with existing metals and settle into the sediments, while a much larger proportion of that is removed by biological oxidation (Jorgensen, 1977). Sulfide is oxidized to elemental sulfur or sulfate by various sulfur-oxidizing bacteria (SOB) that exist in tidal flats. The bacteria are the key organisms which are responsible for the oxidation of inorganic sulfur compounds (Jorgensen and Nelson, 2004; Lenk et al., 2011).

The sulfur cycle of tidal flats in the Ariake Sea had been analyzed through interdisciplinary scientific approaches (Du et al., 2008; Azad et al., 2005). Early information about the bacteria that occupied conserved tidal flats was reported by Liem et al. (2014). They surveyed the diversity and relative abundance of bacterial populations based on 16S rRNA gene. Both SRB- and SOB-related clones are found as major groups indicating active sulfur cycle in the tidal flats. Such culture-independent approach is necessary for providing information on composition of potential sulfur bacteria. However, the role of SOB in oxidation of reduced sulfur compounds in the tidal flat remains unknown.

In the present study, we isolated heterotrophic SOB from a conserved Midorikawa Tidal Flat and observed their attributes in the oxidation of thiosulfate and sulfide. Thiosulfate was used for isolation in this study regarding its stability and abundance in tidal flats. The role of the heterotrophic SOB in the sulfur cycle was discussed.

MATERIALS AND METHODS

Sampling site description

The Midorikawa Tidal Flat is a part of the Midorikawa Estuary, which is located in the western part of Kumamoto Prefecture, Kyushu Island, Japan. At the estuary, fresh water from the Midorikawa River meets seawater from the semi-closed Ariake Sea. The flat becomes submerged by seawater twice a day with a tidal range of 3–4 m (Azad et al., 2005). During low tide, the muddy flat becomes exposed and a number of foraging migratory birds and many burrowing animals, such as mudskippers and crabs, can be observed.

Sampling and isolation procedures

The top 2-cm of sediment was sampled into a sterile 50 mL polypropylene Falcon conical tube (BD Bioscience, Durham, NC, USA) during low tide in June 2013. The sampling tube was kept on ice during trip to the laboratory and stored at 4°C before inoculation. Thiosulfate mineral (TM) liquid medium was used as enrichment medium to grow sulfur-oxidizing bacteria. TM medium was prepared as follows (per liter): 1 g NH_4Cl, 0.1 g $CaCl_2{\cdot}2H_2O$, 10 g NaCl, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 0.8 g $MgSO_4{\cdot}7H_2O$, 3 g $Na_2S_2O_3{\cdot}5H_2O$ (Nacalai Tesque, Kyoto, Japan) in distilled water and 2 mL of 0.5% phenol red as pH indicator. The first three compounds were combined with 1 mL trace metal solution (Robertson and Kuenen, 1983) and autoclaved in 121°C for 20 min and the subsequent components were added after filter sterilization through a cellulose acetate membrane filter (0.2 µm). The final pH of the medium was adjusted to 7.3. A sediment sample of 3 g was transferred to 100 mL TM medium in a 300-mL conical flask. The inoculated flask was plugged with sterile cotton and incubated aerobically at 30°C on a rotary shaker at 120 rpm. Incubation was stopped when the pH of the liquid culture began to drop below 7.0. The liquid culture was then subjected to serial dilution prior to spreading onto TM medium plates containing 1.5% agar and 0.02% yeast extract. The inoculated plates were incubated at 30°C for 7 days under aerobic condition. Morphologically distinct colonies were picked up and streaked onto TM medium plates and they were incubated under the same conditions.

Characterization on oxidizing thiosulfate

Obtained isolates were inoculated into 40 mL of modified TM mediums in 100 mL conical flasks and they were incubated aerobically at 30°C on a rotary shaker at 130 rpm for 10 days. Three modified TM mediums were prepared in combinations of the presence of thiosulfate and pyruvate as an organic carbon source. The first medium namely autotrophic medium was prepared in addition of 0.1% $NaHCO_3$ and absence of pyruvate. The second was mixotrophic medium containing both thiosulfate and 0.1% Na-pyruvate. The third medium was heterotrophic medium and it was prepared in presence of 0.1% Na-pyruvate and omission of thiosulfate. All of the modified mediums contained 0.02% yeast extract. The experiments were replicated two times.

Characterization on oxidizing sulfide

Glass tube was filled with two-layer agar medium and each of which occupied 10 ml. The lower layer contained 1% agar and 400 µl of 10% Na_2S while the upper one contained TM medium with 0.5% agar, 0.1% $NaHCO_3$ and 0.02% yeast extract. Thiosulfate was omitted from the medium leaving sulfide as a sole energy source. Na_2S solution was added in the tube immediately before the lower agar was poured. When the lower agar had turned to solid, 0.5 mL cell suspension in 1% salt solution was transferred on it. Then it was covered by the upper layer agar medium. The inoculated tubes were incubated aerobically at 30°C. The experiments were replicated two times and, as controls, an inoculated medium without Na_2S and a sterile medium with Na_2S were prepared.

PCR amplification of partial 16S rRNA gene

Single colony of purified isolate was picked up from the agar medium using sterile toothpick and transferred directly to the PCR reaction mixture as a template. The universal bacterial primer set of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 518R (5'-GTATTACCGCGGCTGCTGG-3') and AmpliTaq Gold (Applied Biosystems, Carlsbad, CA) were used to amplify the partial 16S rRNA gene. PCR was performed using a T-Gradient Biometra Thermocycler (Biometra, Goettingen, Germany) under the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. To confirm that amplicons were of correct size, the PCR reactions were subjected to electrophoresis on 1.5% (w/v) agarose gels in 1X TAE buffer, gels were stained with ethidium bromide, and reaction products were visualized by illumination with ultraviolet light at 302 nm. PCR products confirmed to be of the correct size were purified using the UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA).

Sequence analysis

The purified PCR products of the partial 16S rRNA genes were sent

Table 1. Growth of five isolates under various conditions to determine ability in oxidizing thiosulfate.

Isolate code	Lithoautotrophic medium ($Na_2S_2O_3$ + 0.1% $NaHCO_3$)		Mixotrophic medium ($Na_2S_2O_3$ + 0.1% Na-pyruvate)		Heterotrophic medium (0.1% Na-pyruvate)	
	pH changes	Turbidity	pH changes	Turbidity	pH changes	Turbidity
MKH02	[a]-1.2	[b]-	-3.6	++	+1.0	++
MKH04	-0.3	+	-0.7	++	+0.1	+/-
MKH16	+0.4	-	+1.4	++	-0.1	-
MKH20	-0.2	-	-0.6	++	+1.2	++
MKH41	+0.8	-	+1.5	++	+0.8	++

[a]+ and – marks, respectively, indicate increase and decrease of the medium's pH after incubation;[b] -, no growth; +/-, poor growth; +, turbidity clearly apparent; ++, stronger turbidity.

to the TaKaRa company to be sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The obtained sequences were uploaded and compared to the GenBank database using the web-based BLAST analysis tools at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/) to analyze their phylogenetic affiliations and identify their closest relatives.

Estimation of heterotrophic thiosulfate-oxidizers abundance in the environment

One-gram sediment sample was subjected to serial dilution in glass tubes containing 9 ml of 1% salt solution. The series included eight 10-fold dilution steps. From each of those, 1 ml suspension was transferred into 40 ml TM liquid medium in 100 ml conical flask containing 0.1% Na-pyruvate as carbon source and 0.02% yeast extract. The medium was prepared in duplicate and thus incubated aerobically at 30°C. The activities of thiosulfate oxidizers were detected as discoloration of the pH indicator.

RESULTS

Isolation of thiosulfate oxidizers

After 7-days incubation, bacterial colonies grew on the all of the agar plates up to 10^5-fold dilution. Prolonged incubation period allowed the colonies to thicken and expand slightly but did not change their numbers. Well-separated colonies were only observed on agar plates with 10^3- to 10^5-fold dilutions. From those plates, colonies were streaked onto fresh agar plates with the same composition. Twenty-five colonies survived on the agar plates. Most of them changed the medium color to yellow while others turn it to pink. As representatives, three acid producers (MKH02, MKH04, and MKH20) and two alkaline producers (MKH16 and MKH41) were chosen for characterization.

Characteristics of the isolates in thiosulfate oxidation

The five isolates distinctly responded to the three conditions of the mediums (Table 1). Isolates of MKH02 and MKH20 could grow in both mixotrophic and heterotrophic medium, but not in autotrophic medium. Another isolate, MKH04, was capable of inorganic carbon fixation and thus it could grow under all conditions as a facultative lithoautotroph. Those mentioned isolates lowered the pH of the mixotrophic medium. In contrast, the remaining two isolates MKH16 and MKH41 increased the pH of the medium during mixotrophic growth and they could not grow in medium lacking organic carbon. Moreover, both of them changed the pH of the mixotrophic medium in a very short time which was less than 12 h while other isolates took 3-8 days to establish growth and decrease the pH of the medium.

Characteristics of the isolates in oxidizing sulfide

Sulfide supplied in the lower agar had diffused to all part of the upper agar within 2 days. The diffusion was recognized by discoloration of the pH indicator to pink. Solution of Na_2S gives alkaline pH since the sulfide ion reacts with H^+ and leaves OH^-. White thin mat was then observed a few millimeters below the surface of upper agar of all isolates. After 10 days of incubation, the mat in the tube inoculated with MKH41 isolate was thicker than that in other tubes. We decided to subculture the mats into fresh agar medium with the same condition. Prolonged incubation of the subcultured isolates faded the mats but again thickened the mat of MKH41 isolate (Figure 1). In controls without addition of Na_2S showed no existence of mats in all isolates. Thin mats appeared in controls with uninoculated medium but they faded away after prolonged incubation. Additionally, discoloration of upper agar to yellow was not observed in all tubes.

Abundance of heterotrophic thiosulfate oxidizers in the environment

In previous analysis, all of the five isolates were capable of growth under simultaneous existence of both thiosulfate and pyruvate and changed the color of the medium during their growth. According to the mentioned characteristics,

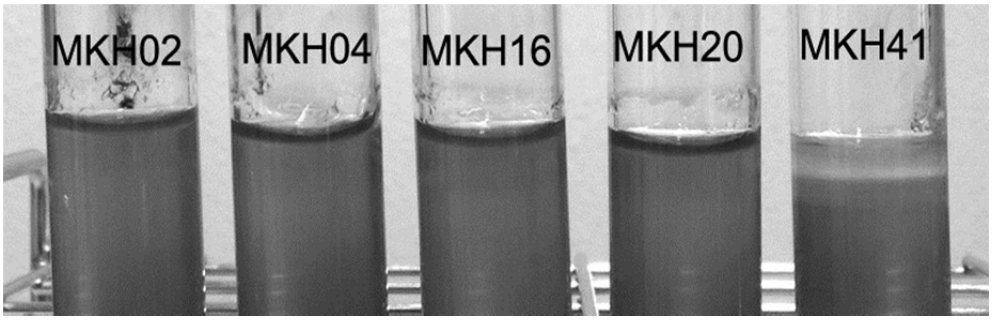

Figure 1. Growth of the heterotrophic SOB in Na_2S agar medium. The white band indicated the bacterial mat of MKH41 isolate that was capable of sulfide oxidation.

Table 2. Identification of heterotrophic thiosulfate-oxidizing bacteria isolated from the Midorikawa tidal flat.

Isolate code	Nearest species/GenBank (Accession No.)	Sequence identity (%)	Class of the nearest species	Origin of the nearest species
MKH02	*Paracoccus homiensis* (NR043733)	99	*Alphaproteobacteria*	Sea-sand sample from South Korea
MKH04	*Paracoccus limosus* (NR109093)	98	*Alphaproteobacteria*	Activated sludge from sewage treatment plant in South Korea
MKH16	*Bacillus jeotgali* (NR025060)	99	*Bacilli*	Korean traditional fermented seafood
MKH20	*Dyella ginsengisoli* (NR041370)	98	*Gammaproteobacteria*	Soil from ginseng field in South Korea
MKH41	*Pseudomonas xanthomarina* (NR041044)	99	*Gammaproteobacteria*	Coastal invertebrate *Halocynthia aurantium* from the Sea of Japan near Russia

we could estimate population number of heterotrophic thiosulfate oxidizers within the tidal flat sediment. The discolorations and turbidities were observed in all of the liquid mediums with dilution up to 10^7-folds after two-week incubation. In all positive dilutions, changes of pH were early observed within 1-2 days of incubation and they all turned to alkaline. Prolonged incubation allowed them to lower the pH leaving the highest two dilutions in alkaline.

Homology analysis

The homology search of 16S rRNA gene showed that the isolates were distributed into three different classes, the *Alphaproteobacteria*, the *Gammaproteobacteria* and the *Bacilli*. Most of the isolates were phylogenetically related to marine microorganism strains already isolated from the regions surrounding Japan. Results of the homology search are presented in Table 2.

DISCUSSION

Characteristics of the isolates in oxidizing thiosulfate and sulfide

Various metabolic types were demonstrated by the isolates regarding thiosulfate oxidation. Isolates of MKH02 and MKH20 grew equally in both heterotrophic and mixotrophic mediums. In those cases, pyruvate served as a sole carbon and energy source. Although the isolates grew under pyruvate, they were capable of oxidizing thiosulfate when it was present. Decrease of the pH of the medium was caused by released of sulfuric acid, the oxidation product of thiosulfate (Friedrich et al., 2001).

In contrast, MKH04 isolate showed growth when it was supplied with thiosulfate. Significant growth was observed in both mixotrophic and autotrophic medium. It indicated that the energy for growth was mainly obtained from oxidation of thiosulfate to sulfate. The isolate could fix inorganic carbon to support its assimilative metabolism when no organic carbon was supplied. In the present study, it was the only one that was capable of autotrophic growth and the growth was demonstrated after 8-day incubation. Coexistence of pyruvate in the medium supported the lithotrophic growth of MKH04 in oxidizing thiosulfate. It was recognized by pH reduction of the medium which was more intense in mixotrophic than in autotrophic growth. Known facultative lithoautotrophs, *Thiobacillus* sp. and *Burkholderia* sp., are reported to produce more biomass and sulfate during mixotrophic than autotrophic growth (Gottschal and Kuenen, 1980; Anandham, 2009).

In the sulfate producers, thiosulfate oxidation is commonly catalyzed by Sox enzyme system and the enzyme is known to be harbored in *Alphaproteobacteria* and *Gammaproteobacteria* (Friedrich et al., 2005). We checked the presence of *sox*B enzyme in the above-mentioned isolates, MKH02, MKH04 and MKH20, by using PCR-based detection. Each of them showed 260 bp amplify-cation of partial *sox*B gene (data not shown) using a serial of degenerate primer sets as described previously (Petri et al., 2001). The positive amplification could suggest the presence of Sox enzyme system in the isolates. Hence the acid producers in this study were likely to use the Sox enzyme in oxidizing thiosulfate.

Another isolate, MKH16, showed no growth on both heterotrophic and lithoautotrophic condition. In contrast, the isolate grew very well under mixotrophic medium. Concomitant existence of organic carbon and thiosulfate appeared to be necessary for its growth. This strain showed a distinguished metabolic pathway in oxidizing thiosulfate by releasing alkaline compound. In alkaline producers, tetrathionate as well as OH^- are released as the main products of thiosulfate oxidation catalyzed by tetrathionate synthase (Muyzer et al., 2013; Sorokin, 2003).

Rises of pH during the growth of MKH41 isolate was showed in all of the three conditions. However, only in autotrophic condition the turbidity was not detected. It was obvious that the growth of the isolate relied on the availability of organic carbon although thiosulfate oxidation was noticed in autotrophic medium. The pathway used in the oxidation of thiosulfate was likely similar with the MKH16 isolate that produced tetrathionate as the final oxidation product. In tetrathionate producers, oxidation of thiosulfate is not an energy-consuming reaction, but instead it releases two electrons for every tetrathionate formed (Podgorsek and Imhoff, 1999). Therefore, a slight increase of pH in autotrophic medium might be a result of thiosulfate oxidation by the inoculated cells but they were unable to grow due to inability of inorganic carbon fixation.

In sulfide oxidation test, MKH41 was the only isolate that survived the successive subculture suggesting that the isolate could oxidize sulfide aerobically. However, the isolate was known to be a heterotroph and no organic carbon was supplied to the medium. The growth might be supported by trace organic carbon existing in the gelling agent. Sulfide oxidation by known tetrathionate producers has been reported and the oxidation product was also end to tetrathionate (Sorokin, 2003).

In the present study, the white mat established under the medium surface might contain elemental-sulfur precipitate which was a product of chemical reaction between tetrathionate and the existing sulfide. Indirect sulfide oxidation by a heterotroph, *Catenococcus thiocyclus*, is observed during growth under an acetate-limited continuous culture. Elemental sulfur and tetrathionate are yielded in the culture (Podgorsek and Imhoff, 1999).

Homology analysis

In the present study, we obtained two isolates grouped in class *Gammaproteobacteria*, the MKH20 and MKH41. In the Midorikawa Tidal Flat, according to culture-independent study performed by Liem et al. (2014), *Gammaproteobacteria* are discovered to be the major group composing more than 20% of the total clones. The class is known to comprise a wide array of autotrophic and heterotrophic SOB (Teske et al., 2000; Meyer et al., 2007) and commonly occupies as the main group in brackish water sediment and coastal marine sediment (Wilms et al., 2006; Asami et al., 2005). The MKH20 and MKH41 were taxonomically related to *Dyella* and *Pseudomonas*, respectively. Several strains of *Dyella* have been isolated as thiosulfate oxidizer from coastal sediment in India (Krishnani et al., 2010) and as PAH degrader in Taiwanese mangrove sediment (Chang et al., 2008). Strains related to *Pseudomonas* reportedly oxidize thiosulfate to tetrathionate under heterotrophic conditions. The genus commonly occupies the interface sulfide-oxygen layer (Sorokin et al., 1999) and a strain was reported to be found in coastal invertebrate at Sea of Japan (Romanenko et al., 2005).

Two other isolates, MKH02 and MKH04 were closely related to genus *Paracoccus*. Such alphaproteobacterial genus is widely found in marine and estuarine sediment and known for its lithotrophy under thiosulfate (Teske et al., 2000; Roh et al., 2009). Alphaproteobacterial group is also known to contribute to the majority of bacterial population after the *Gammaproteobacteria* in Midorikawa Tidal Flat (Liem et al., 2014). As the closest genus for MKH16 isolate, some *Bacillus* strains, have been isolated from saline coastal soil and seawater above hydrothermal vents in South Korea and the North Atlantic, respectively (Siddikee et al., 2010; Rajasabapathy et al., 2014). They exhibit heterotrophic thiosulfate oxidation.

Role of the heterotrophic SOB in the sulfur cycle

In the sulfur cycle of Midorikawa Tidal Flat, MKH41 might oxidize both sulfide and thiosulfate to tetrathionate. The oxidation of thiosulfate in this experiment occurred in a very short time and that possibly occur in the sediment as well. In alkaline producers, oxidation rate of thiosulfate occurs in much faster time than that of sulfide (Sorokin, 2003). Tetrathionate which is released to the environment reacts immediately with the existing sulfide. Such chemical reaction yields thiosulfate and elemental sulfur (S^0). Then the thiosulfate is oxidized back to tetrathionate, hence generating a cycle that consumes sulfide and produces elemental sulfur. This indirect sulfide oxidation may occur in the tidal flat as long as tetrathionate is produced. The tetrathionate producers may compete with SRB that are capable of reduction and disproportionation of thiosulfate to sulfide (Sorokin et al., 1999).

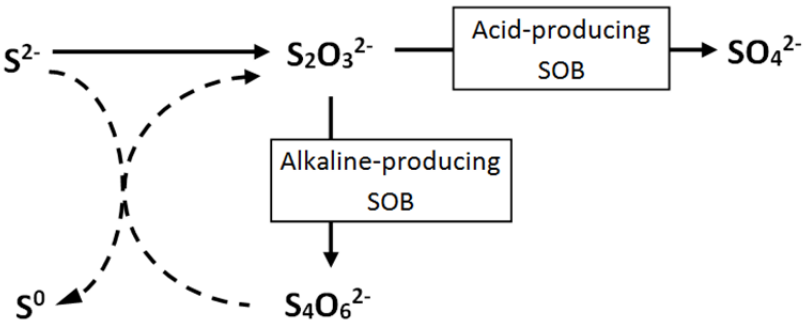

Figure 2. Estimated role of the isolated heterotrophic SOB in the sulfur cycle. Solid arrows indicated the biotic reaction while dashed arrows indicated abiotic reaction. Tangent lines showed that the spontaneous reactions are occurred interdependently.

On the other hand, thiosulfate is oxidizable by the acid-producing SOB. Thiosulfate which is the main product (60%) of sulfide oxidation in tidal flats is partly oxidized to sulfate (Jorgensen, 1990). Heterotrophic sulfate producers obtained from this study was likely to take part in catalyzing such reaction. The possible position of the heterotrophic SOB in the sulfur cycle is summarized in Figure 2. Additionally, populations of heterotrophic SOB in Midorikawa Tidal Flat were estimated in considerable number, up to 10^7 cells/gram of sediment, suggesting their significant role in the sulfur cycle. The high number of the SOB was possibly supported by the tidal flat condition which is rich in organic materials in addition of unlimited supply of oxygen.

Conclusions

Various heterotrophic SOB belonged to *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* were isolated from Midorikawa Tidal Flat. Regarding the final product of thiosulfate oxidation, they were divided into sulfate producers and tetrathionate producers. The heterotrophs oxidized reduced sulfur compounds in presence of organic matter and hence integrated the sulfur cycle with the carbon cycle. Considering their high abundance, their roles could be significant in the conserved tidal flat.

Conflict of interests

The authors did not declare any conflict of interest.

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