Doctoral Thesis

Microbiological Analysis on the Effect of Magnesium Hydroxide for Marine Sediment Remediation Using Molecular Ecological Methods

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CONTENTS

ABSTRACT

The excessive loading of nutrients from the basin has caused the eutrophication and the vast growth of phytoplankton in the estuaries and inner bays. The dead phytoplankton and excessive organic matters accumulated on the surface layer of the sediments. Furthermore, the prosperity of aquaculture fisheries has particularly involved the deterioration of the sediments by the daily feeding. As a result, it accelerates the oxygen consumption in the surface layer of the sediments due to the decomposition of the accumulated organic matter by bacteria. The hypoxic condition in the surface layer of the sediments increased the growth of anaerobic bacteria such as the sulfate-reducing bacteria (SRB), which is responsible for the generation of hydrogen sulfide (H_2S). The toxicity of hydrogen sulfide had strongly impacted on fish and benthic organisms, moreover caused a huge commercial loss for coastal fisheries. In order to control the continual deterioration of the marine sediment environment, several techniques, such as chemical oxidation, iron salts, and increasing pH using effective chemicals are currently used to prevent or control H_2S generation for the remediation of these contaminated sediments. Magnesium hydroxide ($Mg(OH)_2$), as a sediment improving agent, has been widely used for clarification of the sediments under the cage culture field at inner bay in Japan.

In chapter 1, the background and the purpose of the research are described as follows. The excessive loading of nutrients from the basin has caused the eutrophication and the vast growth of phytoplankton in the estuaries and inner bays. The dead phytoplankton and excessive organic matters accumulated on the surface layer of the sediments. Furthermore, the prosperity of aquaculture fisheries has particularly involved the deterioration of the sediments by the daily feeding. As a result, it accelerates the oxygen consumption in the surface layer of the sediments due to the decomposition of the accumulated organic matter by bacteria. The hypoxic condition in the surface layer of the sediments increased the growth of anaerobic bacteria such as the sulfate-reducing bacteria (SRB), which is responsible for the generation of hydrogen sulfide (H_2S). The toxicity of hydrogen sulfide had strongly impacted on fish and benthic organisms, moreover caused a huge commercial loss for coastal fisheries. In order to control the continual deterioration of the marine sediment environment, several techniques, such as chemical oxidation, iron salts, and increasing pH using effective chemicals are currently used to prevent or control H_2S generation for the remediation of these contaminated sediments. Magnesium hydroxide ($Mg(OH)_2$), as a sediment improving agent, has been

widely used for clarification of the sediments under the cage culture field at inner bay in Japan.

The main purpose of this thesis is to determine the remediation effectiveness of $Mg(OH)_2$ on sediment environment and the influences on the bacterial communities in the sediments. in addition, in order to evaluate the inhibition mechanism of $Mg(OH)_2$, the effect of magnesium ion (Mg^{2+}) and alkalization on the generation of H_2S and the microbial community in the sediments is also discussed.

In chapter 2, I review on the biological processes of sulfate reduction in marine environment and those microorganisms associated with this sulfate biological reduction were provided. The field experiments about examining the remediation effectiveness of $Mg(OH)_2$ at cage aquaculture areas and the effect of iron on removing toxic free H_2S from the sediments were also summarized. Finally, the potential future works are addressed.

In chapter 3, the laboratory incubation experiments were conducted to evaluate the inhibition effectiveness of $Mg(OH)_2$ on the generation of acid volatile sulfides (AVS) and H_2S in the sediments. The effect of $Mg(OH)_2$ for the growth of SRB were also investigated by using the real-time PCR method to quantify the target dsrA gene of SRB Grp3 with the specific primers. The results clearly showed that the decrease of the population of SRB Grp3 was strongly related to the decrease in H_2S, and the increase in dissolved organic carbon (DOC) was due to the alkalization by adding $Mg(OH)_2$. However the relation the addition of $Mg(OH)_2$ and the decrease of sulfur reducing activity by other SRBs belonging with Grp1 and Grp2 was not clarified in the experiment yet.

In chapter 4, the next-generation sequencing (NGS) analysis was performed to investigate the effect of $Mg(OH)_2$ for the composition of bacterial communities including all groups (Grp1, Grp2, Grp3) of SRBs in the sediments. Redundancy analysis (RDA) was also performed to illuminate the relationship between the environment factors and the bacterial community composition in the sediments. The RDA results confirmed that adding $Mg(OH)_2$ to the sediment can inhibit the activity of SRB and reduce the generation of sulfide. Meanwhile, the NGS analysis indicated that $Mg(OH)_2$ affects the composition of bacterial communities. In particular, the addition of $Mg(OH)_2$ accelerated the growth of alkaliphilic bacteria.

In chapter 5, the influence of magnesium ion and alkalization on the generation of hydrogen sulfide and the composition of bacterial communities in the sediments were examined. Three treatment groups including the $Mg(OH)_2$ addition group, the sodium hydroxide (NaOH) addition group and the magnesium chloride ($MgCl_2$) addition group were prepared to evaluate whether the inhibition effect of $Mg(OH)_2$ is produced by Mg^{2+} ions or by alkalization. The relationship between environmental factors (pH of the sediments and Mg^{2+} ions content of the

interstitial water) and composition of bacterial community (the dominant bacterial genera) in the sediments was analyzed by a multivariate linear regression model. The NGS analysis and RDA analysis were also performed to prove the results. The results showed that the H_2S content and the copy number of *dsrA* gene in $Mg(OH)_2$, NaOH and $MgCl_2$ addition groups were significantly decreased from the control group. The multivariate linear regression analysis also clearly presented that both the Mg^{2+} ions and the alkalization reduced the generation of H_2S by inhibiting the SRB activity. These results indicated that both alkalization and Mg^{2+} ions are the major factors for the inhibition effect of $Mg(OH)_2$.

In chapter 6, I concluded the major results and also provide the further discussions. Due to the season cycle, the water column in the field sites will show the different characteristics, such as the water temperature, dissolved oxygen (DO), salinity and so on. Due to these differences, the $Mg(OH)_2$ sprayed on the sediments will play different roles in the process of sediment purification. To certificate the purification effects of $Mg(OH)_2$ in the sediments, the field experiments for spraying the $Mg(OH)_2$ to the sediments of fish ponds area last for one year, and measure the chemical and biological parameters in the water column and sediments to analyze the different functions of the $Mg(OH)_2$ during the seasonal cycle.

Keywords: Magnesium hydroxide, Sulfide, Sulfate-Reducing Bacteria (SRB), real-time PCR, Sediment, Next-generation sequencing (NGS), Bacterial communities, Magnesium ion, Alkalization

1 INTRODUCTION

1.1 BACKGROUND

1.1.1 The Situation of Sediments in Eutrophic Marine Areas

The excessive loading of nutrients from the basin has caused the eutrophication and the vast growth of phytoplankton in the estuaries and inner bays. The dead phytoplankton and excessive organic matters accumulated on the surface layer of the sediments. Furthermore, the prosperity of aquaculture fisheries has particularly involved the deterioration of the sediments by the daily feeding. As a result, it accelerates the oxygen consumption in the surface layer of the sediments due to the decomposition of the accumulated organic matter by bacteria. This hypoxic condition in the surface layer of the sediments increased the growth of anaerobic bacteria such as the sulfate-reducing bacteria (SRB), which is responsible for the generation of hydrogen sulfide (H_2S). The toxicity of hydrogen sulfide had strongly impacted on fish and benthic organisms, moreover caused a huge commercial loss for coastal fisheries (Capone & Kiene 1988, Smith *et al*. 1977; Gamenick *et al.* 1996; Vismann 1996; Arndt *et al.* 2013). According to Tyson and Pearson (1991), and Diaz and Rosenberg (2008), the occurrence of bottom water anoxia was high at the coastal zone. Understandably, coastal fisheries could suffer a huge commercial loss from a mass mortality of the fishes. In particular, cage cultures had caused a great deal of loss for coastal fisheries; sediments underneath cage cultures deteriorated faster due to the accumulation of the fish feeds thereby stimulating the H_2S generation on these sediments (Hallare *et al.* 2009). Although the minimization of unconsumed feeds of cage culture has been achieved in terms of both economical reason and conservation of marine environment, the deterioration of the sediment and the generation of H_2S can be induced by the long-term accumulation of the sediments through remains of feeds and excreta of fishes (Devai & De Laune 1995; Avnimelech & Ritvo 2003).

1.1.2 The Sulfur Cycle in Marine Sediments

Sulfur is an element commonly occurring in the environment. It is present in the atmosphere, in the hydrosphere, and in live organisms. There are three main reservoirs of sulfur in its global cycle, i.e. sulfates dissolved in marine waters, sulfates bound in evaporates, and sulfides in clastic marine sediments (Bottrell & Newton 2006). The gas fluxes and sulfates dissolved in the global ocean have been attributed as the main role in the cycling of this microelement (Jasińska *et al.* 2012).

Jørgensen (1990) had proven that sulfur cycling in aquatic sediments involved both reductive and oxidative processes. The anoxic conditions at the bottom of the marine promote the rate of sulfate reduction in sediments, and produce a large amount of hydrogen sulfide. The dissolved hydrogen sulfide can react with iron (II) to form iron sulfides, and be accumulated in the sediments. The dissolved hydrogen sulfide also can be released to the surface layer of the sediments which is a suboxic zone, and then be reoxidized back to sulfate (Lovley & Phillip 1994). These dissolved sulfates in the water column can be assimilated by plants and lead to biosynthesis of sulfur-containing amino acids (Gao *et al.* 2000; Sievert *et al.* 2007). Those bacteria grow in anaerobic conditions also can use dissolved sulfates as the electron accepters and mediate the dissimilative sulfate reduction (Battersby 1988; Boon & Vincent 2003).

Berner (1984) and Lin *et al.* (2000) proved that microbial sulfate reduction plays a key role for bridging the sulfur and carbon biogeochemical cycles, which has been considered as the most significant decomposition pathway of organic matter in marine benthic sediments under anoxic conditions (Jørgensen 1982, Canfield *et al.* 1993). Sievert *et al.* (2007) conclude that the sulfur cycle is closely connected to the cycling of carbon, nitrogen, phosphorus, and iron, and it influences all environmental compartments. Cook and Kelly (1992) also suggested that an enhanced input of sulfate can substantially alter the cycling of elements such as carbon, nitrogen, phosphorus and iron in aquatic sediments.

1.1.3 The Measures of Inhibiting the Generation of Hydrogen Sulfide in Marine Sediments

Several techniques are currently used to prevent or control H_2S generation for the remediation of these contaminated sediments, such as increasing DO concentration, chemical oxidation, iron salts and nitrate salts addition, and increasing pH using effective chemicals (Hobson & Yang 2000; De Lomas *et al.* 2005). Othman *et al.* (2011) and Zhang *et al.* (2008) demonstrated that H_2S produced by SRB in the sewer systems under anaerobic conditions could be inhibited or eliminated through optimization of the environmental parameters: pH, DO and temperature.

Kanaya and Kikuchi (2009) tested a method that controlled sediment H_2S content by forming insoluble FeS after addition of pure iron powder. In laboratory setting, the control treatment contained much more of H_2S and lost the sulfide-reactive Fe^{2+} pool after 20 days of incubation. In contrast, iron addition treatment significantly increased the Fe^{2+} content (including FeS), pH, and the recovered sulfide-reactive Fe^{2+} in the sediment. The field experiments also demonstrated that iron could remove toxic free H_2S from the sediments without any negative effects on macrozoobenthos (Kanaya & Kikuchi 2009).

Magnesium hydroxide ($Mg(OH)_2$) has been used for clarification of the sediments under the cage culture field at inner bay in Japan (Nishno & Kawauchi 2003). Also, it has been applied to inhibit H_2S generation in sewage collection pipelines of municipalities for prevention of corrosion process and odor emission (U.S. EPA 1974; Nielsen *et al.* 1998; Witherspoon *et al.* 2004). Furthermore, Othman *et al.* (2011) investigated the effect of several chemicals on prohibition of H_2S generation in sewage, and declared that $Mg(OH)_2$ could deactivate the sulfide reducing capabilities of SRB by raising the pH of the sewage. Yoshida and Nishino (2005) had examined the inhibition effect of $Mg(OH)_2$ at several cage aquaculture areas for the remediation of the sediment. The results elucidated that after adding $Mg(OH)_2$ to the cage aquaculture area, the pH of the sediment was raised to 8.5, and the total sulfide (TS) in the sediment was gradually lessened. Moreover, Yoshita and Nishino (2005) suggested that spraying $Mg(OH)_2$ to the acidified marine sediments can activate the aerobic bacterium and promote the decomposition of the sludge, inhibiting the growth of SRB and the generation of H_2S.

1.2 OBJECTIVE AND THESIS STRUCTURE

The main objectives of this thesis are to determine the remediation effectiveness of $Mg(OH)_2$ on sediment environment and the influences on the bacterial communities in the sediments.

Chapter 1 gives a brief introduction of the research background, the research objective and the structure arrangement of this thesis.

Chapter 2 provides a general description of the biological processes of sulfate reduction in marine environment and those microorganisms associated with this sulfate biological reduction. The field experiments about examining the remediation effectiveness of magnesium hydroxide ($Mg(OH)_2$) at cage aquaculture areas and the effect of iron on removing toxic free hydrogen sulfide (H_2S) from the sediments are also summarized. Finally, the potential future works are addressed.

In Chapter 3, the laboratory incubation experiments were conducted to evaluate the inhibition effectiveness of magnesium hydroxide ($Mg(OH)_2$) on the generation of acid volatile sulfides (AVS) and hydrogen sulfide (H_2S) in the sediments. The effect of $Mg(OH)_2$ for the growth of sulfate-reducing bacteria (SRB) were also investigated by using the real-time PCR method to quantify the target dsrA gene of SRB with the specific primers.

In Chapter 4, the next-generation sequencing (NGS) analysis was performed to investigate the effect of $Mg(OH)_2$ for the composition of bacterial communities in the sediments. Redundancy analysis (RDA) was also performed to illuminate the relationship between the

environment factors and the bacterial community composition in the sediments.

Chapter 5 focuses on examining the effect of magnesium ion and alkalization on the generation of hydrogen sulfide and the composition of bacterial communities in the sediments. Three treatment groups including the $Mg(OH)_2$ addition group, the sodium hydroxide (NaOH) addition group and the magnesium chloride ($MgCl_2$) addition group were prepared to evaluate the inhibition effect of magnesium hydroxide is produced by magnesium ions or by alkalization. The NGS analysis and RDA analysis were also performed like the Chapter 4 described to prove the results.

Chapter 6 summarizes the major conclusions and provides some discussions of this thesis.

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2 DETERIORATION OF MARINE SEDIMENTS AND THE REMEDIATION: A REVIEW

2.1 DETERIORATION OF SEDIMENTS OF CLOSED INNER BAY-AN EXAMPLE OF OMURA BAY

2.1.1 The Geographical and Environmental Conditions of Omura Bay

Omura Bay (32°57′0″N, 129°52′30″E) is a bay located in the center of Nagasaki prefecture (Figure 2-1). The bay measures about 11 km east-to-west and 26 km north-to-south. The surface area is about 320 km^2 and the length of the shoreline is about 360 km. Compared to its size, the bay is relatively shallow with an average water depth is about 15 m and the maximum depth is 54 m. It is distinguished by its extremely closed topographical characteristic. Actually, the bay appears as an inland sea on maps which is surrounded by land in all directions, and has only two narrow connections to the open sea, therefore, the nutrients and pollutants input from rivers are difficult to exchange quickly with the surrounded sea areas. Due to this extremely closed characteristic, Omura Bay is subject to the influence of external loads from rivers and surrounding agriculture lands, and it is considered to be a typical inner bay that easily suffer to organic water pollution and eutrophication (Takahashi *et al.* 2009; Taguchi *et al.* 2014). In particular, excessive inflow of nutrients causes the occurrence of eutrophication and leads to the outbreak of phytoplankton. Therefore, even though the inflow of organic matter is reduced, organic pollution is to proceed indirectly through the photosynthesis of phytoplankton.

2.1.2 Influence of Eutrophication on Sediments

As a result of eutrophication, large amounts of dead bodies of phytoplankton accumulated on the surface of sediment and led to the deterioration of water and sediment quality. Since the increase of organic matter in the sediments stimulate the activity of bacteria, as a result, the dissolved oxygen (DO) in the sediments or the overlaying water is consumed. In summer, due to the water temperature of the sea surface layer is usually higher than that of the bottom layer, the formation of thermocline will block the vertical transport of oxygen which supplied from sea surface or supplied by photosynthesis of the phytoplankton. Therefore, the decrease of oxygen transporting to the bottom layer of the sea reduces the ability of oxygen supplication. However, the increased organic matter stimulates the activity of aerobic bacteria and accelerates the biodegradation of those organic matter accumulated on the surface sediments. As a result,

the oxygen dissolved in the sediments is rapidly consumed and induces the formation of hypoxic, even the anoxic condition in the bottom layer.

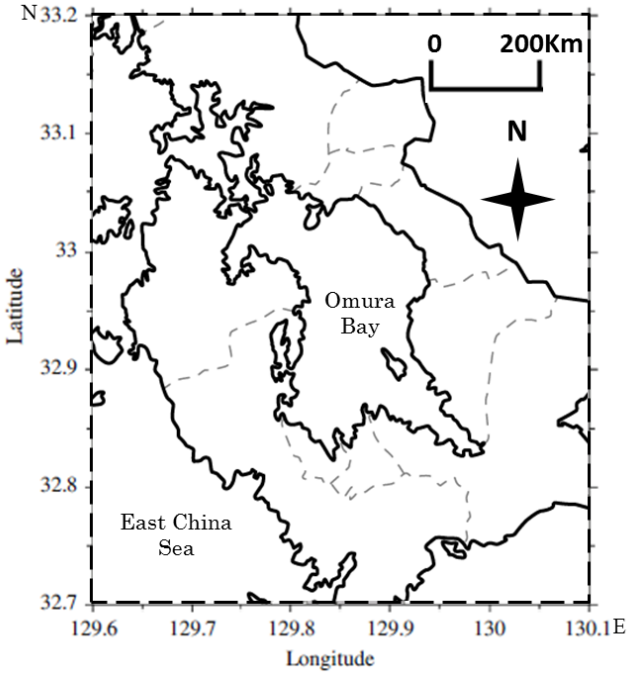

Figure 2-1 Full view of Omura Bay.

Taguchi *et al.* (2014) carried out a precise numerical simulation about the Rise and fall of the hypoxic water mass in Omura bay using his 3D eco-hydrodynamic model. They resulted in the abstract as follows (Taguchi *et al.* 2014), "The result shows that increased stratification during July-September induces and develops hypoxia in the bottom layer from June to mid-August by limiting vertical transport of oxygen. The benthic hypoxia reaches the highest stage in mid-August, then begins to decline gradually afterwards but lasts until early October. The recovery process of oxygen turned out to be closely related with change in the pathway of oceanic water intrusion into the central basin. After September, the oceanic water with rich oxygen begins to flow directly into the central basin, contributing toward alleviation of the hypoxia. Episodic wind event enhances vertical mixing and destroys the bottom hypoxic water quickly but only intermittently: hypoxia comes back and covers the seabed again immediately after the wind ceases. It is only a strong northerly wind caused by a tropical storm in the early stage of October that leads it to a termination every year."

This oxygen-free condition is generally greater impact on the sediment itself. Especially in the coastal zone which the occurrence of bottom water anoxia is high (Tyson & Pearson 1991; Diaz & Rosenberg 2008) and the aquaculture fisheries are prosperity and deeply involved the

deterioration of the sediments. The feed remains and feces from aquaculture fields and the dead bodies of phytoplankton and zooplankton caused by eutrophication will settle out of the water column and accumulate on the surface layer of sediment. These deposited organic matters can be decomposed and used as carbon and energy sources by aerobic bacteria in the sediments. As a result, the bacterial activity is promoted and the dissolved oxygen in the surface layer of sediment and their overlaying water is consumed. This hypoxic condition will threaten the survival of benthic organisms, deteriorate sediment environment and water quality and even induce anaerobic reaction mediated by anaerobic bacteria in the surface layer of sediments. Hallare *et al.* (2009) reported that the sediments under the water area of cage cultures deteriorated faster than those no fish culture areas. The main reason is attributed to the accumulation of the fish feeds which accelerated the oxygen consumption in the sediments by bacterial decomposition, and then stimulated the activity of SRB and the generation of H_2S. The toxicity of H_2S strongly impacted on fish and benthic organisms, their dead bodies accumulated on the sediments again and enhanced the deterioration of the sediments.

2.2 SEDIMENTARY SULFUR CYCLING AND SULFATE REDUCTION

2.2.1 Sulfur Cycle in Marine Sediments

Sulfur has proven to be one of the 15 basic elements that build the Earth and constitute 1.93% (by weight) of our planet (Pempkowiak 1997). It is present in the atmosphere (i.e. sulfates bound in evaporates), in the hydrosphere (i.e. sulfates dissolved in marine waters) and in the biosphere (i.e. sulfides accumulated in marine sediments), which represent three main reservoirs of sulfur in its global cycle (Bottrell & Newton 2006). Although these three sulfur reservoirs constitute a much smaller portion of the total cycled sulfur, they play a significant role in the transport of sulfur in the all environmental compartments. Many researchers also estimated that the sulfur cycle has a close connection with the cycling of carbon, nitrogen, phosphorus and iron in the terrestrial and aquatic ecosystem. Jørgensen (1982) and Canfield *et al.* (1993) suggested that the microorganism-mediated sulfate reduction plays a key role in bridging sulfur and carbon biogeochemical cycles, and also be considered as the most major pathway of mineralizing organic matter in marine sediments under anoxic conditions. Berner (1989), Wang *et al.* (2008) and Fan *et al.* (2012) also proved that the sedimentary sulfur transformation in marine area tightly connected to the global sulfur cycles and the carbon mineralization.

The global sulfur cycle which the sulfur compounds present in the atmosphere, biosphere, hydrosphere and the sediment layer of oceanic lithosphere is presented in Figure 2-2. Dimethyl

sulfide (DMS) and carbonyl sulfide (COS) are two of the most important organosulfur compounds present in the atmosphere. DMS originates and emits primarily from DMSP, a precursor of DMS synthesized by the photoautotrophic species of marine planktonic algae (Gao *et al.* 2000; Iverson *et al.* 1989). Sievert *et al.* (2007) demonstrated that maximum around 2% of DMSP will be released into the atmosphere as DMS which diffuses readily from water due to its poor solubility. DMS in the marine atmosphere is oxidized to sulfur-containing compounds such as sulfur dioxide and sulfuric acid. Malin (2006) suggested that sulfuric acid has the potential to create aerosols which act as cloud condensation nuclei to influence the cloud formation. That is, the massive production of atmosphere DMS may have a significant impact on the Earth's climate (Bates *et al.* 1987, Pham *et al.* 1996, Norris 2003). Sulfur dioxide is another product from the oxidation of DMS. It has been considered as the most dangerous air pollutant, which may cause respiratory ailments and have a negative impact on human circulatory systems (Jasińska *et al.* 2012). Furthermore, most sulfur dioxide in the atmosphere will be oxidized to sulfur trioxide and then forms sulfuric acid when it reacts with water, which gets deposited on the Earth's surface as so-called acid rain. This deposition pathway is very important in the case of the terrestrial part of hydrosphere, which as the direct input source of sulfur from the atmosphere into the seas and oceans. Carbonyl sulfide (COS) is another very important source of forming aerosols and sulfur dioxide in the atmosphere after undergoing photooxidation (Pham *et al.* 1996). This sulfur compound is formed during biodegradation of dissolved organic matter (Uher & Andreae 1997) and photochemical reaction between sulfur and carbon (Ferek & Andreae 1984). It diffuses very readily from water into the air after be synthesized.

In the hydrosphere, marine environment is the most important and largest reservoir of sulfur. Except the direct input of sulfur from the atmospheric deposition mentioned above, weathering and leaching of minerals, oxidation of sulfides and riverine inflow also are the most important sources of sulfates in marine waters (Jasińska *et al.* 2012). Marine phytoplankton plays a key role in sulfur cycle in the natural environment. Gao *et al.* (2000) and Sievert *et al.* (2007) demonstrated that most sulfates dissolved in the water column are assimilated reduced to sulfide by planktonic algae, and then bound into organic acids to synthesize sulfur-containing amino acids such as cysteine and methionine. These organic sulfur compounds undergo biological or chemical decomposition and finally convert to hydrogen sulfide (H_2S). On the other hand, these planktonic algae also can use exogenic amino acids to synthesize DMSP, which is the main emission source of DMS (Gao *et al.* 2000). The dissolved sulfates in the water column also can be reduced to sulfide via dissimilatory pathway, which is mediated by anaerobic bacteria using

organic compounds as electron donors. These bacteria are mostly belonging to the class Delta-proteobacteria that is called sulfate-reducing bacteria (SRB). Hydrogen sulfide (H_2S), as a product of the dissimilatory sulfate reduction, can diffuse to the entire sediment layers under the anoxic condition and also can be reoxidized to sulfate via chemical reaction (such as reacts with oxygen, nitrate, ferric oxide and manganese oxide) and via microbial processes (such as sulfide-oxidizing bacteria (SOB) oxidize sulfide into sulfate for obtaining energy) (Jørgensen 1977; Thamdrup *et al.* 1994; Schenau 2002; Bottrell & Newton 2006; Sievert *et al.* 2007). Jørgensen (1982) also demonstrated that around 75-90% of sulfides in the marine environment undergo reoxidation. Šukytė *et al.* (2002) suggested that the products of sulfide oxidation are different depend on the environmental pH value, which elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}) are basic products, while the possible products are hyposulfite ($S_2O_4^{2-}$), hyposulfate ($S_2O_6^{2-}$) and tetrathionate ($S_4O_6^{2-}$) when the pH less than 7. Carbonyl sulfide (COS), which formed by biodegradation of dissolved organic matter, also can be hydrolyzed to hydrogen sulfide, and then hydrogen sulfide can be reoxidized to sulfate by chemical or biological pathway.

In the marine sediments, the accumulation of large amounts organic matter on the surface layer of the sediments stimulate the microbial activity and induce the available dissolved oxygen at the interface of sediments and overlaying water is rapidly exhausted. Under the anoxic condition, hydrogen sulfide is predominantly produced by sulfate-reducing bacteria. Due to hydrogen sulfide has a high affinity for binding with metals, when the amount of reactive metal ions in the sediments are sufficient for metal sulfide formation, the heavy metals and hydrogen sulfide then both are immobilized in the sediment. These products commonly are poorly soluble metal sulfides that can be considered as the main factor of controlling the bioavailability of heavy metals in the sediments and removing dissolved hydrogen sulfide from the pore water of sediments.

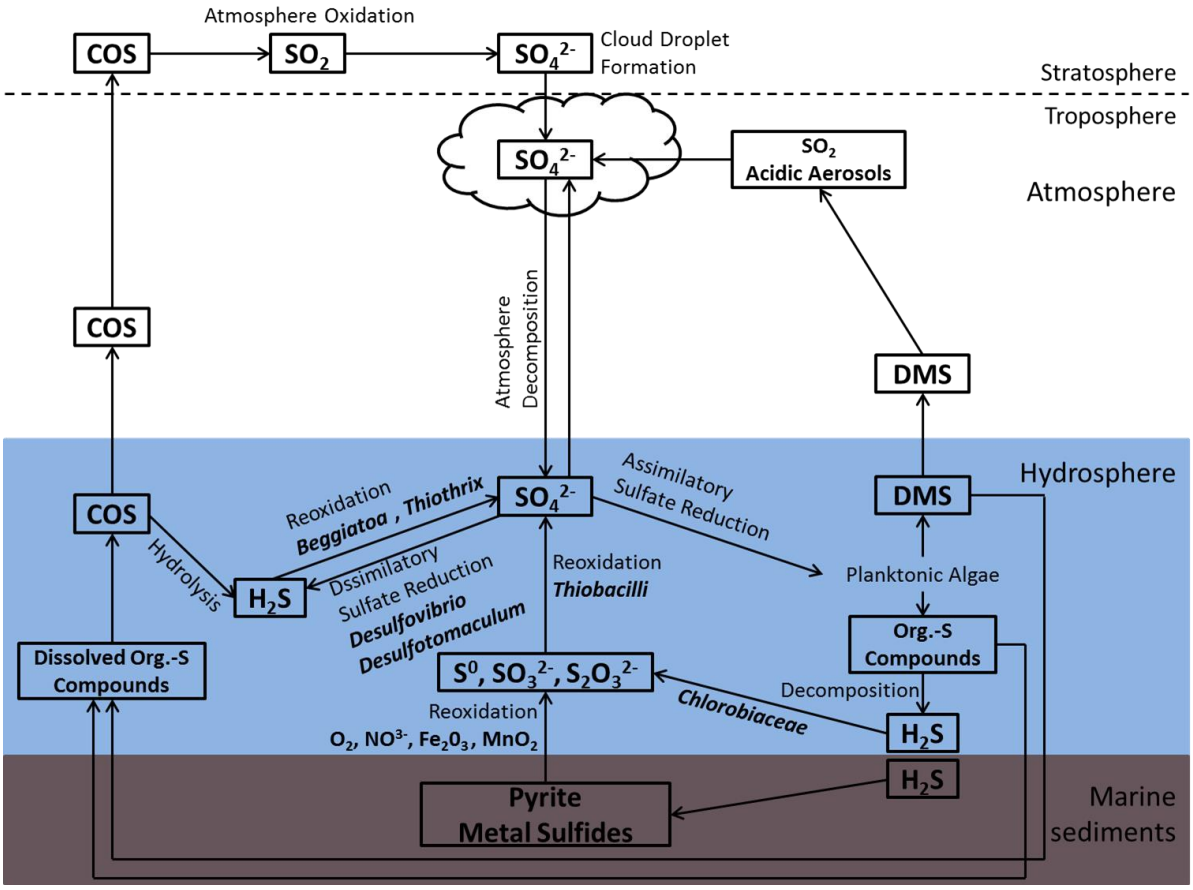

Figure 2-2 The sulfur cycle (modified according to Jasińska *et al.* (2012), Andreae & Jaeschke (1992) and SCOPE (1993)). DMS: dimethyl sulfide; COS: carbonyl sulfide; pyrite: FeS_2; sulfate-oxidizing bacteria (SOB): *Beggiatoa*, *Chlorobiaceae*, *Thiothrix* and *Thiobacilli*; sulfate-reducing bacteria: *Desulfovibrio*, *Desulfotomaculum*.

Consequently, amount of hydrogen sulfide are produced and some of them will be transferred to the atmosphere, the others are either re-oxidized at the oxic-anoxic interface, or precipitated in the form of iron sulfides. In organic rich and iron limited sediments, organic sulfur can be transformed to hydrogen sulfate via bacterial respiratory reduction, or to sulfate and hydrogen sulfide via bacterial disproportionation (Thamdrup *et al.* 1994). In the presence of iron and manganese compounds, the few of produced hydrogen sulfide can be precipitated to iron monosulfide, and then be ultimately converted to pyrite. The remaining will be re-oxidized chemically or by bacterial processes (Böttcher 1999).

2.2.2 The Biochemical Processes of Sulfate Reduction

Muyzer & Stams (2008) suggested that sulfate is a poor electron acceptor for microorganisms due to its unfavorable redox potential (SO_4^{2-}/SO_3^{2-}: E^0=-516 mV). It is quite electronegative

that cannot be reduced by intracellular electron mediators present in sulfate reducers. In order to make sulfate become a useful electron acceptor, it must be activated firstly by an ATP-sulfurylase enzyme for forming adenosine 5'-phosphosulfate (APS) and pyrophosphate, and then APS reductase reduces APS to sulfite, finally sulfite reductase reduces sulfite to H_2S and releases it. The processes mentioned above are the typical pathway of dissimilatory sulfate reduction in three two-electron reduction steps (Fitz & Cypionka 1990). Assimilatory sulfate reduction is an energy-consuming process, it reduces sulfate to elemental sulfur on a small scale for use in biosynthesis by many organisms; moreover, this process is mediated by phosphoadenosine-5'-phosphosulfate (PAPS) reductase, which is formed from phosphorylation of APS by APS kinase. NADPH reduces PAPS to sulfite, and finally sulfite is reduced by sulfite reductase to H_2S for assimilation into organic S compounds (Carbonero *et al.* 2012). The differences between these two pathways are that assimilatory sulfate reduction is performed by autotrophic organisms for biosynthesis of organic sulfur compounds, while dissimilatory sulfate reduction is preceded via anaerobic pathways for generation bacterial energy (Grein 2013).

Dissimilatory sulfate reduction seems to be a strictly anaerobic process as all the microbes capable of reducing sulfate to sulfide only grows in environments devoid of oxygen (Andreae & Jaeschke 1992). This kind of anaerobic bacteria as so-called sulfate-reducing bacteria (SRB) is phylogenetic diversity. Muyzer & Stams (2008) summarised that most of the information on the diversity of SRB in natural ecosystem has been obtained by the use of marker genes such as 16S rRNA gene and by the use of functional genes such as *dsrAB* gene. Larsen *et al.* (2000) found that the *dsrABD* operon was formed by *dsr* genes, which *dsrA* encodes α structural subunit and *dsrB* encodes β structural subunit. The *dsrD* gene encodes a conserved unknown protein apparently restricted to sulfate-reducing species. Müller *et al.* (2015) summarized that the DsrAB-type dissimilatory (bi)sulfite reductase is a key microbial enzyme in both reductive and the oxidative steps of the biogeochemical sulfur cycle. It also has been confirmed that playing a central role in catalyzing six electron reduction of sulfite to sulfide during anaerobic respiration with sulfate, sulfite or organo-sulfonates as terminal electron acceptors. Dahl *et al.* (1993) also indicated that *dsrAB* enzymes are heterotetramer proteins with a structure of two α subunits and two β subunits ($\alpha_2\beta_2$), which are encoded by *dsrA* and *dsrB* genes respectively. The *dsrA* gene and *dsrB* gene are organized a single operon with *dsrA* preceding *dsrB* and are normally used as *dsrAB* gene. Nowadays, *dsrAB* gene has been widely used for molecular phylogenetic relation of SRB and detection of SRB by PCR from the environment.

2.3 THE GENERATION OF HYDROGEN SULFIDE AND RELATED BACTERIA IN SEDIMENT ECOSYSTEM

2.3.1 The Generation of Hydrogen Sulfide in Sediments

Although the dissolved oxygen (DO) in the overlaying water is sufficient, the interior of sediments is an anaerobic and reductive atmosphere environment (oxidation-reduction potential (ORP) is negative which depend on the hydrogen potential reference). Sulfate-reducing bacteria (SRB) can use sulfate ion as an oxidant to oxidize organic matter and generate energy, meanwhile sulfur ion and hydrosulfide ion as terminal products are produced. But since the reductive state of sediments, sulfur ion and hydrosulfide ion will transform to dissolved hydrogen sulfide. When free ferrous ions (Fe^{2+}) are present in sediments, hydrogen sulfide will immediately combines to them and form stabilized iron sulfide (FeS). In addition, a part of dissolved hydrogen sulfide can diffuse into the surface layer of sediments and be reoxidized to sulfate ion again by sulfur oxidizing bacteria. Therefore, only little amount of hydrogen sulfide can diffuse into the overlaying water.

On the other hand, when the overlaying water is anoxic condition, the surface layer of sediment will become reductive state, which will promote the activity of SRB and generate hydrogen sulfide. When the ferrous ions (Fe^{2+}) are present, iron sulfide (FeS) will be formed in the surface layer of sediments. Moreover, since the iron ions and sulfate ions can be supplemented by the inflow from open sea, if the organic matter is sufficient, iron sulfide will continue to be produced and finally lead to an increase of the total sulfide content in sediments. Jørgensen (1983a) indicated that most of the sulfate reduction takes place in the upper 10cm of sediments, and the highest seasonal dynamic variation of sulfate reduction is observed within a short distance of the sediment-water interface. In the surface layer of sediments, the rate of microbial sulfate reduction is primarily controlled by the quality and availability of organic matter, sulfate concentration, temperature, microbial species diversity and oxidation pathways of organic matter, salinity, sediment depth, sedimentation rate, bioturbation, bioirrigation (Jørgensen 1982; Gagnon *et al.* 1996; Donahue *et al.* 2008). Furthermore, Berner and Westrich (1985) had proven that only 10% of the sulfide produced by microbial sulfate reduction can be permanently deposited in the sediments, and most of them precipitate in the form of iron sulfides (FeS and FeS_2). While, the others 90% of the reduced sulfide will be re-oxidized to sulfate or reduced sulfur compounds, such as sulfite, thiosulfate and elemental sulfur. Jørgensen (1982) also proposed that about 25% to 50% of the dissolved oxygen consumption in the sediments is involved in the re-oxidation of sulfide.

In addition, hydrogen sulfide has a strong toxicity and greatly impact on most of benthic

organisms and fish. Although some benthic organisms such as worms have a certain degree of tolerance to anoxic condition, there is a high possibility of migration or death if they suffered H_2S. Worms are known to promote the mineralization of sediments by bioturbation, Kersey-Sturdivant *et al.* (2012) carried out an in situ observation, and found a result as follows, "Some infaunal burrowing and movement continued when the water column was severely hypoxic or anoxic. Prior to the onset of anoxia several spionid polychaetes, *Paraprionospio pinnata*, were observed at the sediment surface with their characteristic palps extended into the water column at a DO of 0.1 mg L^{-1}. As DO concentration declined further to anoxia it appeared that *P. pinnata* continued to burrow through the sediment and flocculent bacterial mat." Therefore, their death will weaken the purification ability (organic miniralization) of sediments by bioturbation, accelerate the contamination of organic matter and the formation of the anoxic water mass, finally lead to a further deterioration of sediments, even fall into a vicious circle.

2.3.2 Microbial Ecology of Sulfate-Reducing Bacteria (SRB)

The sulfate-reducing bacteria (SRB) are found in several different phylogenetic lines, where Deltaproteobacteria accounts for the largest group of SRB and most of the known dissimilatory sulfate reducers are classified in this class, such as *Desulfovibrio*, *Desulfosarcina*, *Desulfomonas* and *Desulfobacterium* (Battersby 1988; Boon & Vincent 2003). There are also three genera of Archaea known to be capable of sulfate reduction: *Archaeoglobus*, *Thermocladium* and *Caldivirga*. Most of SRB grow in anaerobic conditions using low molecular weight organic compounds as electron donors such as lactate, acetate and propionate. These organic compounds are primarily fermentation products of bacterial degradation of carbohydrates and proteins, and also are the most important source of carbon and energy for SRB (Parkes *et al.* 1989; George *et al.* 2008; Pfennig & Biebel 1986). However, certain species from genus *Desulfovibrio* only can oxidize organic compounds incompletely to acetate. In the genus *Desulfobacter*, some species which can oxidize organic compounds completely to carbon dioxide are also exist. On the other hand, sulfur-reducing bacteria such as genus *Desulfuromonas*, can use element sulfur as electron donor to oxidize organic compounds and produce hydrogen sulfide.

2.4 COUNTERMEASURE STRATEGIES FOR REMEDIATION OF MARINE SEDIMENTS

The interaction between deteriorated sediments and anoxic marine sediment environment further aggravates the deterioration for each other. Furthermore, a large amount of hydrogen

sulfide generated in the anaerobic sediments indicates a possibility of facing a high toxic risk in benthic organisms and a high level of degradation of this sediment system. Therefore, the countermeasures from the viewpoints of environmental conservation, securing of marine resources and reduction of damage to aquaculture are required. Among them, eliminating the hypoxic even the anoxic condition in the bottom layer of marine is the major work. Nowadays, strategies for source control and natural recovery, such as removal and containment, removal and treatment, *in situ* capping, and *in situ* treatment have been employed as a form of environmental remediation in marine area, especially in estuary and inner bay which coastal aquaculture are popular (Reis, Lodolo & Miertus 2007). These sediment management strategies are generally divided into *in-situ* remediation and *ex-situ* remediation technologies which include mechanical method, physicochemical method and biological method.

2.4.1 *Ex-situ* sediment remediation technologies

2.4.1.1 Mechanical Methods (Dredging, Tillage and Flow promotion)

Dredging, sediment tillage and flow promotion are typical mechanical remediation methods for sediments. Dredging is by removing the deteriorated sediments to achieve the purification effect and also can suppress the formation of anoxic water mass. The dredging technologies and the sediment characteristic are the two important factors which will determine the magnitude of dredging environmental impacts (Manap & Voulvoulis 2015). Tillage can transport oxygen into the sediments by plowing; meanwhile it can activate the activity of microorganisms and promote the decomposition of organic matter in sediments. Flow promotion is an effective method in principle. The propulsion of tidal current can agitate and move sediments, dissolved oxygen also can be transported simultaneously to the bottom layer of marine with the tidal current to improve the anoxic condition. Therefore, in some cases this method can be done at low energy and low cost by using topography and tidal current. These methods mentioned above have actual effect for transporting oxygen to the bottom layer and eliminating the formation of anoxic water mass in the water column. However, several disadvantages also restrict the application of these methods, such as the mechanical instruments and regular enforcement are necessary for sediment dredging and tillage, flow promotion is only effective in a narrow water area and it requires a large amount of energy input in marine area. The main advantages and disadvantages of these methods are summarized in Table 2-1.

Table 2-1 The main advantages and disadvantages of *ex-situ* sediment remediation technologies .

Advantages	Disadvantages
● Can agitate or remove contaminant sediments; ● Can transport oxygen into the sediments, meanwhile activate the microbial activity and promote the decomposition of organic matter in the sediments; ● Can eliminate the anoxic water mass present in the water column.	● Require large construction equipment to conduct dredging operations and transporting dredged sediments; ● Require off-site disposal for dredged sediments (need dredged sediments reach an adequately dried state; need approved solid waste management facility or landfill); ● Have a large environmental footprint which create a variety of impacts to the local community; ● Noise and in-water turbidity generated from sediment dredging and support equipment create an impact to people and organisms living in the surround areas.

2.4.2 *In-situ* sediment remediation technologies

In-situ sediment remediation technologies include *in-situ* containment and *in-situ* treatment which generally involves addition and mixing of biological organisms (Biological Method) or immobilization reagents with contaminated bottom sediment (Physicochemical Method). The primary advantage of such technologies is treating the sediments in place, resulting in less re-suspension of contaminated sediments, reducing the handling operations and the exposure of contaminated sediments. Finally, these techniques are also relatively simple and inexpensive (Reis, Lodolo & Miertus 2007). The main advantages and disadvantages of *in-situ* sediment remediation technologies are summarized in Table 2-2.

2.4.2.1 Biological Method

Microbial agent and bioturbation are commonly used methods to purify the sediments within the biological method. Microbial agent is consisted by a group of microorganisms with high decomposition activity. Spraying them to the sediments to promote biodegradation via their high decomposition activity is the main purpose of this method. But as a new member of the local ecosystem, these microorganisms are considered as foreign species, which is possible to disturb the original ecosystem. Bioturbation is similar with mechanical tillage, but use benthos organisms to instead the mechanical instruments. The activities of benthos organisms agitate the sediments and promote decomposition of the sediments (Kersey-Sturdivant *et al.* 2012). However, most benthos organisms have a low activity in anaerobic condition, that is, bioturbation is basically effective only in an aerobic environment.

Table 2-2 The main advantages and disadvantages of *in-situ* sediment remediation technologies.

Advantages	Disadvantages
Biological methods: ● Can promote microbial activities and their decomposition capabilities; ● Can promote enzymatic production to degrade or transform contaminants to less toxic or non toxic forms; Physicochemical methods: ● Can destroy contaminants completely or alter the form of the contaminants to optimize process conditions for other treatment processes; ● Chemical treatment processes can provide oxygen and nutrients for microbial use and oxidation of organopollutants ; ● Chemical reagents such as iron, steel slag and magnesium hydroxide can suppress sulfide formation by alkalization as well as phosphorus absorption effect.	Biological methods: ● Introducing exotic species is possible to destroy the local ecosystem; ● Environmental conditions for various stages of the biodegradation process is difficult to adjust; Physicochemical methods: ● Lack of process control, excessive addition of chemical reagents will stimulate the production of organic matter and enhance the deterioration of sediment quality; ● Lower treatment efficiency than *ex-situ* treatment; ● Require a long-term implementation for regular adding chemical reagents; ● Limited experience with *in-situ* treatments.

2.4.2.2 Physicochemical Method

Physicochemical method such as spraying the sediment improvement agent has been carried out before. Calcium nitrate, steel slag (Sato *et al.* 2003) and magnesium hydroxide (Nishno & Kawauchi 2003) are commonly used ones. Nitrate ions can directly chemically oxidize and decompose the organic matter in sediments, and calcium ions can absorb phosphoric acid. The remaining nitrate ions will be eliminated as nitrogen gas since the denitrification reaction by microorganisms is carried out promptly in anaerobic sediments. Due to organic matter are used in this denitrification reaction, decomposition of organic matter in sediments is more effective. However, excessive addition of calcium nitrate will stimulate the growth of phytoplankton due to excess nutrients. As a result, eutrophication is accelerated and leads to deterioration of water quality and sediments.

Iron and steel slag also has been examined in purification effect. It mainly includes iron oxide, calcium oxide and magnesium oxide, which is considered to have the effect of suppressing sulfide formation by alkalization as well as chemical oxidation and phosphorus adsorption effect like calcium nitrate.

Magnesium hydroxide is considered to reduce the activity of sulfate reducing bacteria by

weak alkalization and has adsorption effect of phosphorus. As a result, spraying magnesium hydroxide as sediment improvement agent is possible to avoid killing benthic organisms and promoting remediation. From the field tests such as aquaculture farms, it was confirmed that spraying magnesium hydroxide could inhibit the generation of sulfide to improve the deteriorated sediments, promote revision of benthic organisms such as shellfish and disappear odor caused by hydrogen sulfide. However, the results of field tests also show that regular spraying of magnesium hydroxide and a long-term implementation are necessary until a clear effect appears. Moreover, the influences of long-term spraying on sediment ecosystem and the sustainability of the effect from magnesium hydroxide are unknown.

2.5 REMEDIATION OF SEDIMENTS BY MAGNESIUM HYDROXIDE

Regarding the effect of magnesium hydroxide on the sediments, it is considered that magnesium hydroxide has inhibition effect on sulfate-reducing bacteria, since the significant reduction of odor produced by hydrogen sulfide in fish farms has been reported. Actually, Nishino & Kawauchi (2003) had examined the inhibition effect of $Mg(OH)_2$ at several cage aquaculture areas for the remediation of the sediment. The results elucidated that after adding $Mg(OH)_2$ to the cage aquaculture area, the pH of the sediment was raised to 8.5, and the total sulfide (TS) in the sediment was gradually lessened. Moreover, they also concluded that a long-term implement of magnesium hydroxide addition treatment was necessary. Figure 2-3 showed that the initial concentration of total sulfide in sediments was around 1.1 mg g^{-1}, it took almost 3 years decreased to 0.4 mg g^{-1} (Nishino & Kawauchi 2003). Figure 2-4 also showed a clear decrease of total sulfide in magnesium hydroxide addition area compared to control area without addition (Nishino & Okinaga 2002). The initial concentration of total sulfide was over 3 mg g^{-1}, indicated an extremely high contamination degree in sediments. It took around 4 months decreased to less than 1 mg g^{-1} (Figure 2-4). Moreover, Nishino & Okinaga (2002) also estimated the number of sulfate-reducing bacteria by using bacterial pure culture with agar medium. As a result, the number of sulfate-reducing bacteria clearly decreased in $Mg(OH)_2$ addition area compared to non-addition area (Table 2-3). The results mentioned above suggested that the pH of sediments was increased to 8 or more by spraying $Mg(OH)_2$ to the sediments, as a result, the number of sulfate reducing bacteria and the content of sulfides decreased.

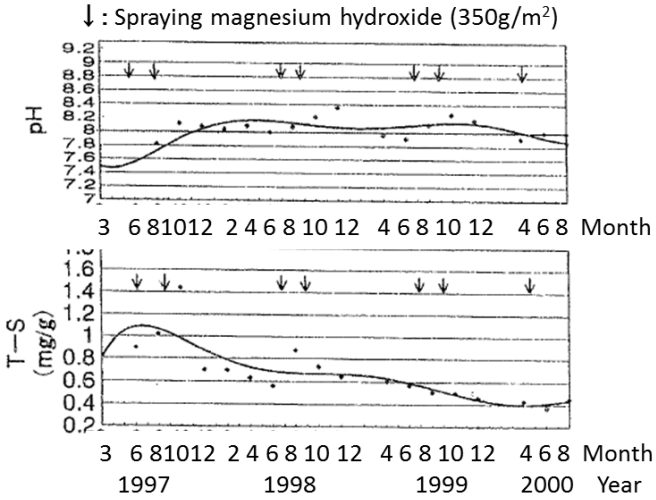

Figure 2-3 The temporal changes of total sulfide (T-S) and pH of the marine sediments in fish ponds (March 1997 to August 2000) (Nishino & Kawauchi 2003).

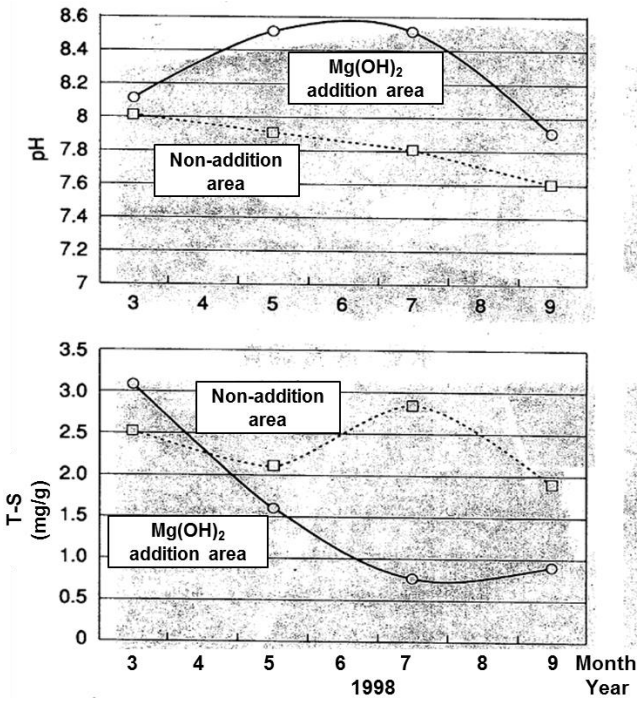

Figure 2-4 Changes of pH and total sulfide (T-S) in the marine sediment of the pearl farms (March to September 1998) (Nishino & Okinaga 2002).

Table 2-3 The temporal changes of sulfate-reducing bacteria (SRB) in the marine sediments of fish farms (May to September 1998) (Nishino & Okinaga 2002).

Sampling Date	Sampling Site	pH of Sediments	Sulfate-Reducing Bacteria (cells/wet-g)
21 May	Non-add.	7.9	60.0×10^2
	Add.	8.5	6.4×10^2
15 July	Non-add.	7.8	11.0×10^2
	Add.	8.5	1.9×10^2
9 September	Non-add.	7.6	5.3×10^2
	Add.	7.9	1.7×10^2

Non-add.: non-addition area; Add.: $Mg(OH)_2$ addition area.

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3 ROLE OF SULFIDE REDUCTION BY MAGNESIUM HYDROXIDE ON THE SEDIMENT OF THE EUTROPHIC CLOSED INNER BAY

3.1 INTRODUCTION

During summer days, enclosed estuaries and inner bays may possibly suffer from eutrophication due to the stratification of the water resulting for the concentration of dissolved oxygen (DO) below the surface to lessen. This phenomenon can trigger the production of hydrogen sulfide (H_2S) through sulfate-reducing bacterial activity creating an anoxic condition to the benthic region (Valdemarsen *et al.* 2009). Sulfate-reducing bacteria (SRB) are a diverse group of anaerobic microorganisms, which are responsible for the H_2S production (Stahl *et al.* 2002). Identification and characterization of new SRB has been facilitated by using the dissimilatory sulfite reductase (DSR) gene as a target for phylogenetic analysis. The DSR gene product catalyzes the reduction of sulfite to sulfide which is a key step in sulfate respiration and is therefore considered highly conserved among sulfate-reducers (Karkhoff-Schweizer *et al.* 1995).

Magnesium hydroxide ($Mg(OH)_2$) has been used for clarification of the sediments under the cage culture field at inner bay in Japan (Nishno & Kawauchi 2003). Yoshida and Nishino (2005) had examined the inhibition effect of $Mg(OH)_2$ at several cage aquaculture areas for the remediation of the sediment. The results elucidated that after adding $Mg(OH)_2$ to the cage aquaculture area, the pH of the sediment was raised to 8.5, and the total sulfide (TS) in the sediment was gradually lessened. Moreover, Yoshita and Nishino (2005) suggested that spraying $Mg(OH)_2$ to the acidified marine sediments can activate the aerobic bacterium and promote the decomposition of the sludge, inhibiting the growth of SRB and the generation of H_2S.

Consequently, bench scale laboratory experiments using the collected marine sediment to determine the remediated effectiveness of $Mg(OH)_2$ addition on sediment quality was being conducted in recent studies. The first objective of this chapter was to evaluate the inhibition effectiveness of magnesium hydroxide ($Mg(OH)_2$) on the generation of acid volatile sulfides (AVS) and hydrogen sulfide (H_2S) in the sediments collected at Omura Bay. Lastly, the effect of $Mg(OH)_2$ for the growth of SRB were also investigated.

3.2 MATERIALS AND METHODS

3.2.1 Study Site and Sample Collection

Omura Bay is an inner bay located at the center of Nagasaki Prefecture, Japan. The bay is regarded as a typical example of a eutrophic inner bay. It is strongly affected by the external load from the river, the surrounding farmland and organic water pollution. The sediment sample was collected from the southeast water area of the Omura bay (32˚84′N, 129˚98′E) which is 5.4km (269˚5′W) far from the Isahaya city. The study was consisted of two experiments: experiment-1 and experiment-2. For experiment-1, samples were collected on September 12, 2013. For Experiment-2, samples were collected on February 24, 2014. Each sediment samples were split into two equal parts, with one stored for DNA extraction at -80 ºC and the other stored for further chemical analysis at -4 ºC.

3.2.2 Experimental Setup

3.2.2.1 *Experiment-1 Reduction effect of AVS in sediment*

For experiment-1, there were two treatments involved: no addition treatment (control group) and $Mg(OH)_2$ addition treatment (treatment group). The preparation for the treatment involves removing of visible small litters such as pebbles, shells, small benthic animals and plant debris from the collected sediments. After mixing thoroughly, 170 g of sediments were put in a small plastic container (3.5 cm in width, 3.5 cm in depth and 7 cm in length). For the treatment group, clear water (Ube Materials Co., Yamaguchi, Japan) which has $Mg(OH)_2$ granules of 2mm diameter was sprayed on the top of the sediments (Figure 3-1). Three sediment containers were prepared for each treatment (three replicates).

The two treatments (three replicates) then were put into reactors which mimicked field conditions. A reactor is a rectangular-shaped container (45 cm in length, 30 cm in width and 35 cm in depth) filled with 20 L of sea water, collected from Omura bay, and a nitrogen gas aerated at 100 mL min^{-1} (Figure 3-2a & b). The sea water in the reactor was kept at 26ºC using AQUA COOLER SLIM202 constant-temperature water circulator (MARUKAN, NISSO Department, Osaka, Japan) during the 20-day incubation.

For experiment-1, the moisture content of the five parallel sediment samples in each container was measured every 4 hours at 105ºC using a laboratory oven (MOV-212F-PE Panasonic, Osaka, Japan). The dissolved oxygen (DO) was measured daily by Firesting O_2 fluorescent oxygen sensor (PyroScience GmbH, Aachen, Germany). The initial pH values were measured for the three sediment samples (three replicates) which were uniformly mixed, before spraying $Mg(OH)_2$ by using the pH Electrode, ELP-038 with 3 mm diameter pH sensitive glass

tips (TOA DKK, Tokyo, Japan). After the 20-day incubation, the pH of each sediment samples was measured in the same way. In addition, DOC of the interstitial water and AVS in the sediment were also measured. These parameters were measured for three sediment containers of each sample with three replicates.

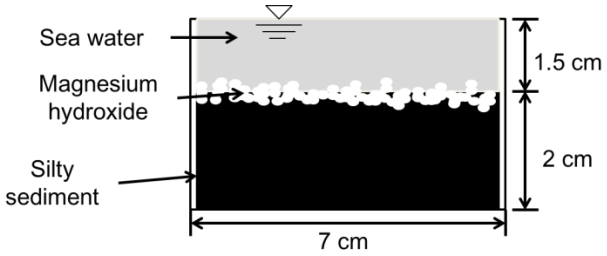
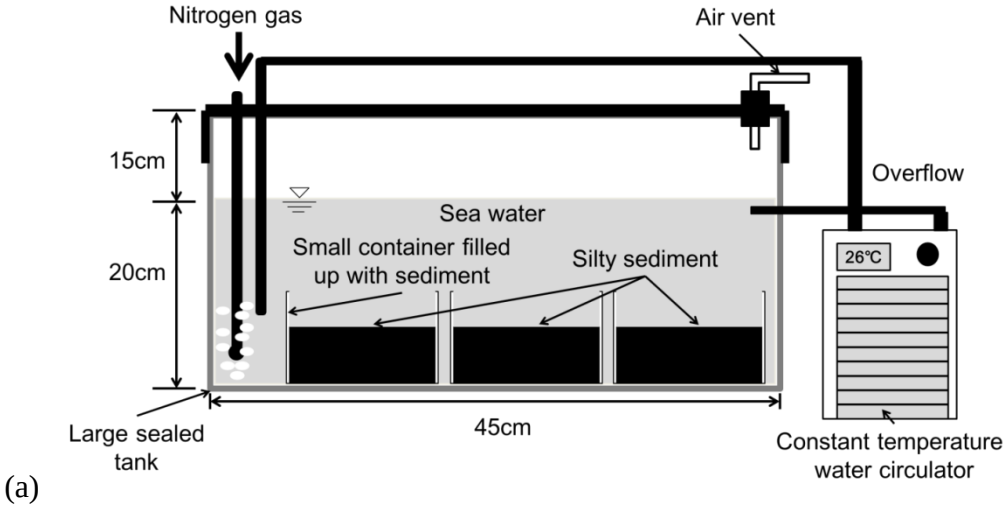
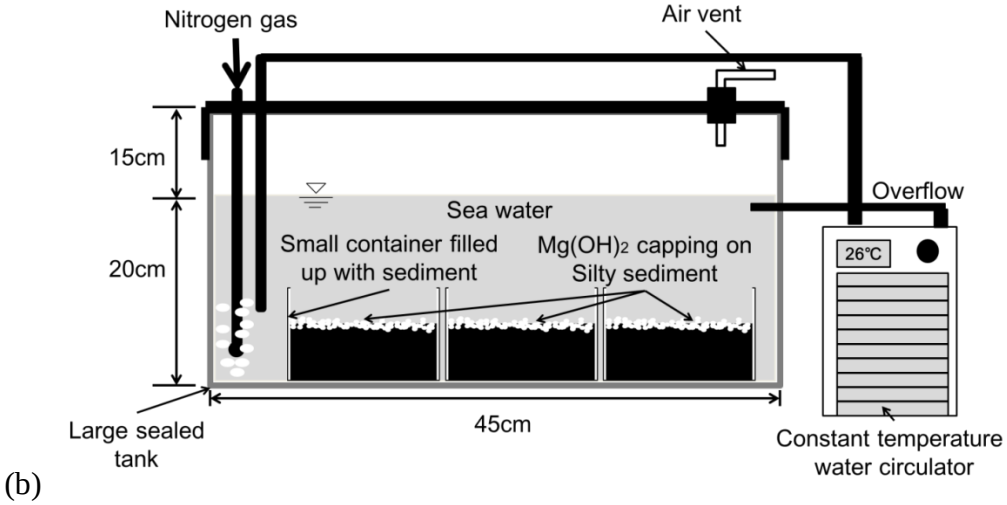


Figure 3-1 Magnesium hydroxide ($Mg(OH)_2$) capping on silty sediments



(a)



(b)

Figure 3-2 Schematic figure of the equipment for Experiment-1. (a) Control group without $Mg(OH)_2$, (b) Treatment group with $Mg(OH)_2$ granules scattered on the sediments

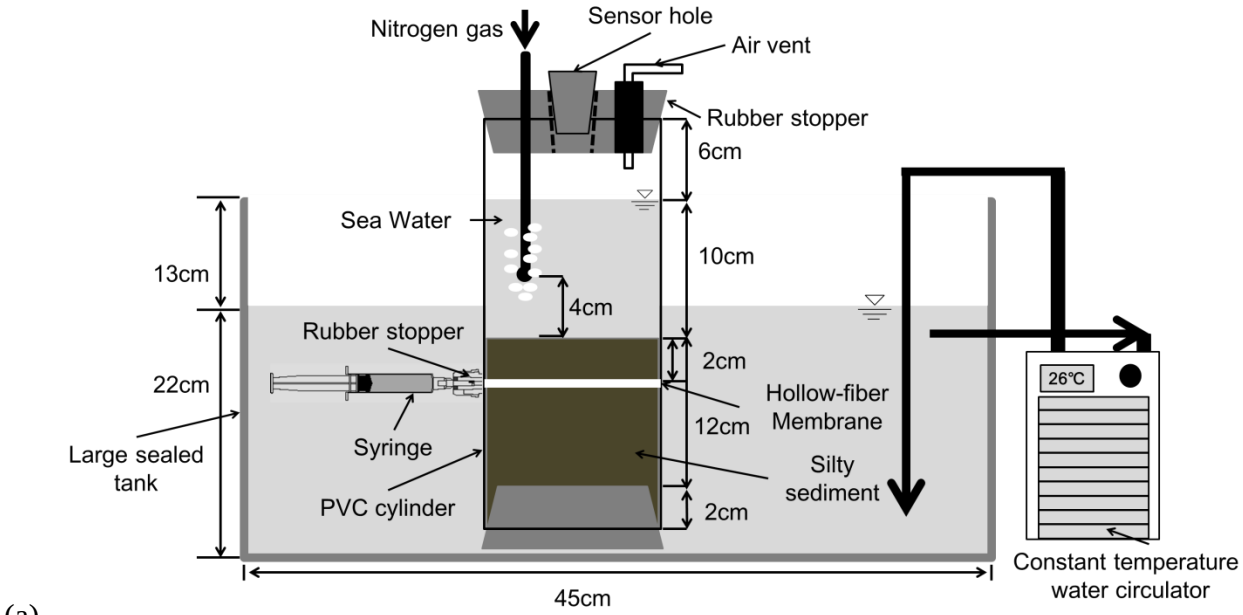
3.2.2.2 *Experiment-2 Inhibition effect of H_2S in sediment*

Polyvinyl chloride (PVC) cylinders with a 10cm inner diameter and a height of 30cm were prepared for the sediment column experiment shown in Figure 3-3a & b. The top and the bottom of the PVC cylinder were sealed with two rubber caps. The cylinder was filled with sediment, which was uniformly mixed (same as in experiment-1), up to 12 cm height from the bottom of the cylinder. The rest of the column was filled with seawater collected from Omura bay. It was then aerated 4 cm above from the surface with 100 mL min^{-1}pure nitrogen gas to prevent the re-suspension of the sediment at the surface.

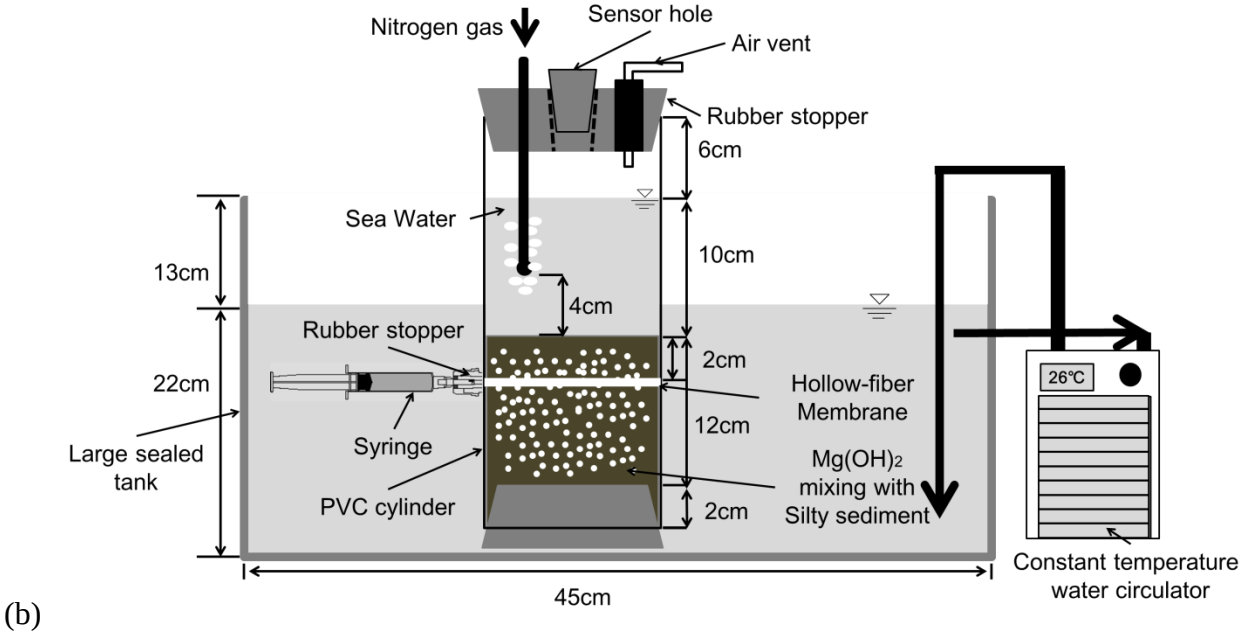
No addition treatment and $Mg(OH)_2$ (0.5kg m^{-2}) addition treatment were prepared. Duplicate cylinders were prepared for each group. The cylinders were incubated in a water chamber and kept at 26ºC using the AQUA COOLER SLIM202 constant-temperature water circulator (MARUKAN, NISSO Department, Osaka, Japan).

In experiment-2, polytetrafluoroethylene (PTFE) hollow-fiber microfiltration (MF) membrane (Sumitomo Electric Industries, Osaka, Japan) with the pore size of 0.2 μm and outer diameter of 2.5mm was used to collect the interstitial water in the sediment to measure H_2S. Currently, this kind of membrane was widely used in MBR (Membrane Bio Reactor) for wastewater treatment. It was set at the 2cm depth from the sediment surface presented in Figure 3-3a & b. One end of the hollow fiber MF membrane was sealed with epoxy adhesive. The other end, demonstrated in Figure 3-3a & b, was perforated to the outside of the cylinder, which was connected to the syringe of 1 mL volume with a needle (Terumo Co., Tokyo, Japan) and was sealed with epoxy adhesive. The syringe had been always connected to the hollow fiber membrane during the incubation to prevent the surrounding seawater to invade the sediment column. Then, hydrogen sulfide (H_2S) in the collected interstitial water was measured by methylene blue method described in the later section.

For experiment-2, DO was measured by YSI ProODO Optical Dissolved Oxygen Instrument (YSI Incorporated, Ohio, USA). The pH was measured at 1 cm depth under the sediment surface in each cylinder, and used the same pH electrode in experiment-1. In addition, DNA extraction and real-time PCR for the sediment samples before and after the incubation were conducted to quantify the copy number of *dsrA* gene of SRB in sediment.



(a)



(b)

Figure 3-3 Schematic figure of the equipment for Experiment-2. (a) Control group without $Mg(OH)_2$, (b) Treatment group with $Mg(OH)_2$ granules mixed in the sediments

3.2.3 Chemical Parameters of the Sediments

3.2.3.1 *Acid Volatile Sulfides (AVS)*

Acid volatile sulfides (AVS) was used as a surrogate parameter of TS (Rickard & Morse 2005). AVS was measured in a sulfide detection tube (Detector Tube No. 201H; GASTEC, Kanagawa, Japan) as follows: two grams of a sample was mixed with 2 mL of 18N sulfuric acid, which was continuously pumped into the tube until the color of the tube changed. Five

parallel measuements for each sample were carried out. The detail of the measurement method was in accordance to the manual kit provided by the manufacturer.

3.2.3.2 *Dissolved Organic Carbon (DOC)*

Thirty (30) mL of each sediment sample was put into a 50 mL plastic conical centrifuge tubes (Sigma-Aldrich Corp., St. Louis, MO, USA), it was then centrifuged for 20 minutes in 4,000 rpm at 25ºC to collect the supernatant (interstitial water) (Vanderborght & Billen 1975; U.S. EPA 2001). The DOC in the interstitial water was measured via non-purgeable organic carbon (NPOC) measurement method in accordance with the standard measurement methods (JIS K0101:1998 Testing Methods for Industrial Water) by using TOC-L equipment (Shimadzu, Co., Kyoto, Japan). The samples (10-40 µl) were introduced by direct aqueous injection onto a platinum-on-alumina catalyst heated to 680ºC in a quartz reaction tube. Prior to analysis, the samples were treated with 20% phosphoric acid and sparged with CO_2-free air for 90 seconds to remove inorganic carbon (Sugimura & Suzuki 1988). Since the average DOC value was obtained automatically by three repeating measurement for a sample was the only available value, it was used as the averaged value for each sediment samples in three replicates.

3.2.3.3 *Hydrogen Sulfide (H_2S)*

Four (4) mL of interstitial water from sediment was gently collected by the syringe, then the interstitial water H_2S was quantified by methylene blue method (Yano 2002). One (1) mL of 0.23 M Zinc acetate (Zn^{2+}) was immediately added to the collected interstitial water to deposit H_2S, HS^- and S^{2-}. Zinc sulfide (ZnS) deposition was re-dissolved by adding 0.4 mL of 0.0096 M N, N-dimethyl-p-phenylenediamine ($C_8H_{12}N_2$) and then 0.2 mL of 0.020 M $FeCl_3$ solution was added to generate methylene blue (adopted methylene blue method). The standard solution of H_2S (1000mg L^{-1}) was prepared by adding 0.75g sodium sulfide ($Na_2S{\cdot}9H_20$) into a 100 mL oxygen-free water, a product of pure nitrogen gas bubbled through MilliQ water for 30 minutes in advance. The standard solution was diluted to several concentrations, and then added the same N, N-dimethyl-p-phenylenediamine solution and $FeCl_3$ solution as in the procedure for the sample measurement. After the methylene blue was generated, the samples were analyzed by the UV-VIS spectrophotometer UV1800 (Shimazu Co., Kyoto, Japan) at 668 nm.

3.2.3.4 *DNA Extraction*

In experiment-2, uniformly mixed sediments of each group was divided into three subsamples each weighing ca. 0.8g (wet weight). Microbial DNA was extracted from each

subsample before and after the 7-day incubation (Alain *et al.* 2011). The extraction was carried out using the FastDNA Spin Kit for Soil (MP Bio Japan, Tokyo, Japan) and followed the manufacturer's instructions.

3.2.3.5 *Real-Time Quantification PCR*

The copy number of *dsrA* gene of SRB was quantified in each extracted DNA template using real-time PCR SYBR method. Present study stated that *dsrA* gene was amplified with the Grp3 forward primer (CTGCGAATATGCCTGCTACA) and the Grp3 reverse primer (GGGGCARCCGTCGAACTTG) (Spence *et al*. 2008). The standard curve for total SRB enumeration using Grp3 primer set was generated using an artificially synthesized DNA. The linear range of detection for real-time PCR assay was at least five orders of magnitude from 1.0×10^5 to 1.0×10^9 copies per PCR (Figure 9). The artificially synthesized DNA, including a binding region for both primers, was inserted into pUC19 plasmid (Sma I site) and was used as the standard DNA of SRB for the quantification by real-time PCR. The sequence of insertion DNA fragments were as follows:

5'-
GTCTGGGCCAGTCCCGCTGCGAGCTGCGAATATGCCTGCTACGACACCCAAGACAT
GTGCTGACCATGGACTATCAGGACGAAATCCACCGTCCGGCCTTCCCCTACAAGTT
CAAGTTCAAGTTTCAAGTTCGACGGTTGCCCCAACGGTTGCGTAGCTGCCGTTCC
GACTTTGCCGTTAGCCGTTATCGGCACCTGGACA-3' : 210bp. The construction of the plasmid with the synthesized DNA was performed by Sigma-Aldrich Corporation (Sigma-Aldrich Corp., St. Louis, MO, USA).

Amplification by real-time PCR was carried out using a Thermal Cycler Dice® Real Time System II (Takara Bio, Kusatsu, Shiga, Japan). All of the reactions were performed in a 20 μL volume of 96-well plates where triplicates of DNA template of all samples and the standard DNA templates (1.0×10^9, 1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5 copy mL^{-1}) were prepared. The PCR reaction mixture contained 10 μL of a THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Osaka City, Osaka, Japan), 6pmol of each primer, 1μL of BSA solution (51 μg μL^{-1}) and 1μL of 10-fold dilution of the extracted DNA. The temperature cycle consisted of an initial denaturation step of 95°C for 30sec , followed by 45 cycles each subjected to an denaturation of 95 °C for 10sec (denaturing) and 62°C for 30 sec (annealing and extension), respectively.

3.2.4 Statistical Analysis

The data were expressed as mean (error bar = standard deviation (SD)). The statistical

difference was determined by two-sided one-way ANOVA using statistical software R (version 3.3.1), and $P<0.05$ was considered significant. The one-way ANOVA test was adopted the Welch's method (1951) since it doesn't require the equal variance assumption between the groups (Moder 2010).

3.3 RESULTS

3.3.1 The Results of Experiment-1

The 20-day anoxic incubation was done by aerating nitrogen gas and by keeping the DO stable around 0.3-0.4 mg L^{-1} for the control group, while maintaining around 0.2-0.3mg L^{-1} of DO with $Mg(OH)_2$ for the treatment group. The pH, AVS and DOC were measured at the start of the experiment before any addition of $Mg(OH)_2$. The measured values were observed as the common initial values for the treatment group and the control group. The initial value of pH of the sediment was 7.43 ±0.08, and the AVS content was 2.48 ±0.28 mg dry-g^{-1}. The obtained average value of the water content (66.09%) in the collected sediment sample was used for the calculation of AVS as per dry gram weight of sediment.

After the 20-day incubation, the pH of the treatment group increased to 8.25 ±0.13, which was significantly higher (P=0.002) than the initial pH value (Figure 4). The content of AVS of the treatment group significantly reduced to 2.13 ±0.18 mg dry-g^{-1} (P=0.046) (Figure 5). On the other hand, there is no significant changes around the pH of the control group (Figure 4), and the obtained AVS value (2.43 ±0.21 mg dry-g^{-1}) was not significantly different (P=0.73) with the initial value (Figure 5). The DOC of the treatment group (40.1 ±1.87 mg L^{-1}) and control group (29.0 ±5.93 mg L^{-1}) increased from initial DOC (15.4 ±0.40 mg L^{-1}), whereas significant increase was observed in treatment group rather than the control group (P=0.0017 and P=0.39, respectively) (Figure 6).

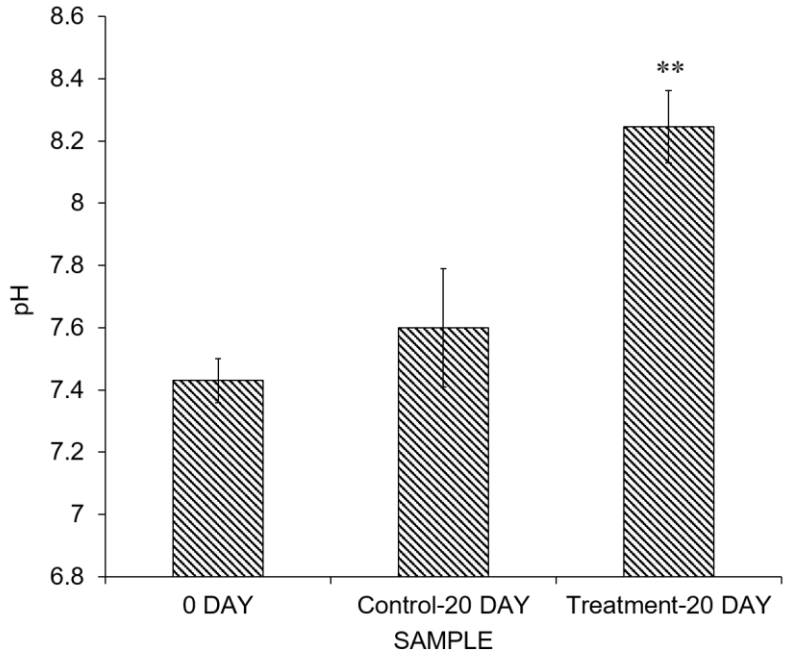


Figure 3-4 Changes of sediment pH of each group. The data are presented as mean (error bar = SD), n=3. Means labeled with ** (two asterisk) are significantly different to the samples of 0 DAY based on one-way ANOVA test ($P \leq 0.01$)

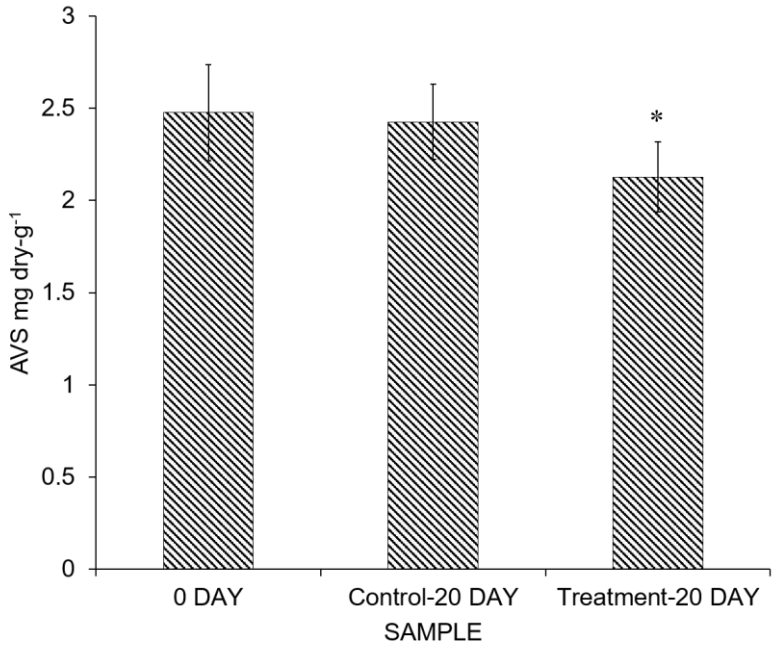


Figure 3-5 Changes of Acid Volatile Sulfides (AVS) concentration in the sediments of each group. The data are presented as mean (error bar = SD) of five observations for 0 DAY samples, Control-20 DAY and Treatment-20 DAY samples. Means labeled with * (one asterisk) are significantly different to the samples of 0 DAY based on one-way ANOVA test ($P \leq 0.05$)

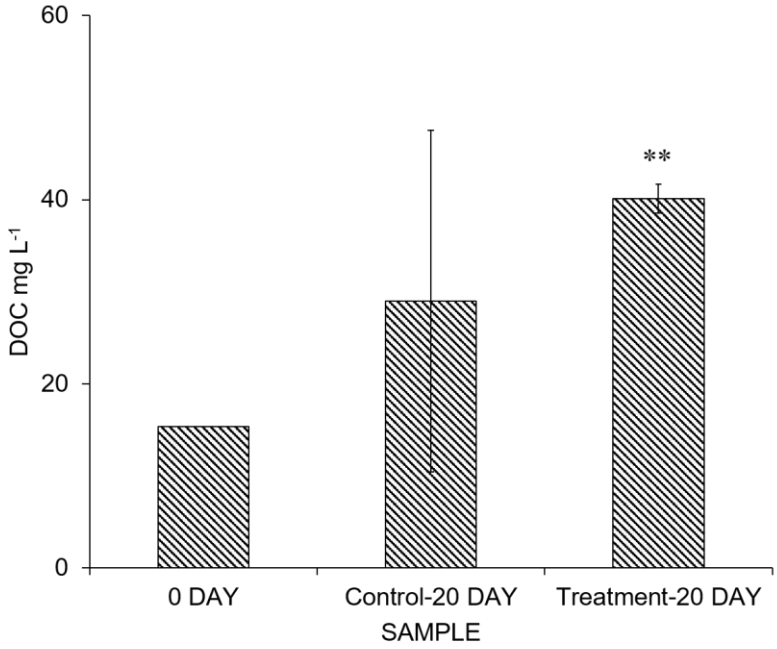


Figure 3-6 Changes of Dissolved Organic Carbon (DOC) concentration in interstitial water of each group. The data are presented as mean (error bar = SD) of three observations for 0 DAY, Control-20 DAY and Treatment-20 DAY samples. Means labeled with ** (two asterisk) are significantly different to the samples of 0 DAY based on one-way ANOVA test ($P \leq 0.01$)

3.3.2 The Results of Experiment-2

Dissolved oxygen (DO) in all cylinders were maintained around 0.4 mg L^{-1} during the 7-day incubation. In this experiment, the initial pH value of the sediment was measured for each column on the starting day after putting it up to the PVC cylinder. The pH value of the treatment group had slightly decreased (P=0.0056) from 8.94 ±0.05 (as 95% confidential interval: 8.82~9.07) to 8.53 ±0.09 (as 95% confidential interval: 8.31~8.76) by the addition of $Mg(OH)_2$ as shown in Figure 7. The decreasing trend of pH from 7.54 ±0.01 to 7.07 ±0.04 was also observed in the control group (P=0.0021) indicating that the fermentation of organic acid phases (sediment and water) might be a principal factor in terms of regulating the pH levels.

The measurement result of H_2S was unstable in comparison with the other parameters in this experiment even though the method used was design to carefully oxidize H_2S as long as possible. The H_2S concentration of the treatment group was at 0.073 ±0.03 mg L^{-1} after 1-day incubation. After the 7-day incubation, the average H_2S concentration of treatment group was significantly decrease (P=0.0017) (Figure 8). However, no significant difference (P=0.39) was observed between the H_2S concentration of the control group sediment after 1-day

incubation and that of after 7-day incubation (Figure 8).

The results of real-time PCR reaction of *dsrA* were obtained for each DNA templates. The standard line (R^2=0.9941) for the quantification of *dsrA* gene was illustrated in Figure 9. Since the DNA sample was extracted before starting the incubation, "0 DAY" was used as the initial time (Figure 10). The copy number of the initial *dsrA* gene (1.19 ±0.37 ×10^8 copy mL^{-1}) in the control group was almost same as the copy number (1.11 ±0.18 ×10^8 copy mL^{-1}) after the 7-day incubation (*P*=0.69). On the other hand, the copy number of the treatment group (0.48 ±0.05 ×10^8 copy mL^{-1}) was significantly lower after the 7-day incubation (*P*=0.0019).

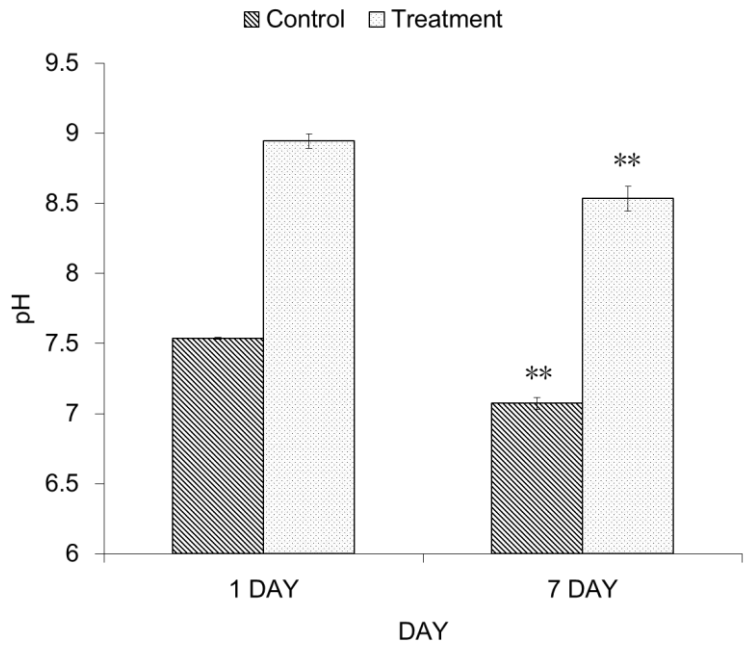


Figure 3-7 Changes of sediment pH of each group. Data are presented as mean (error bar = SD). The asterisk marks above the both bar graphs at "7 DAY" show the statistical significant level as follows (n=3): * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$)

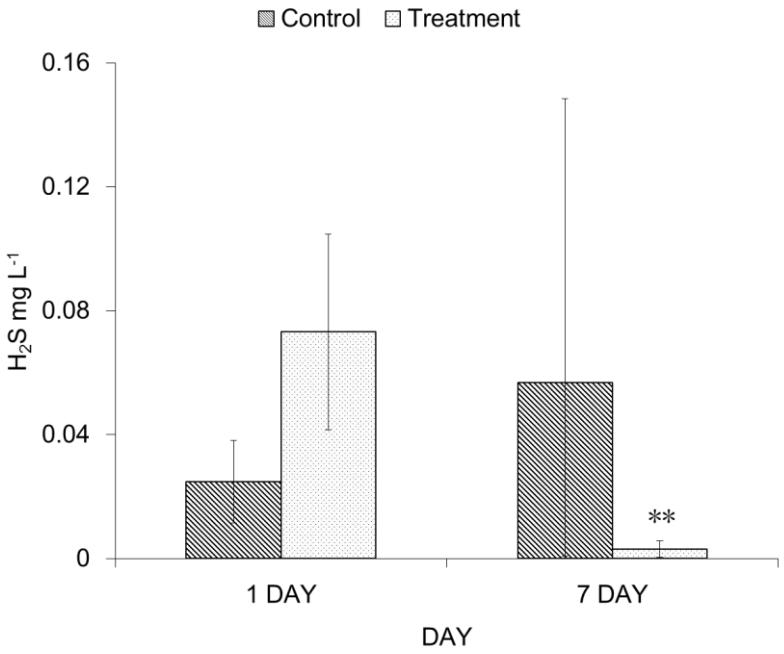


Figure 3-8 Changes of H_2S concentration in interstitial water of each group. Data are presented as mean (error bar = SD). The asterisk marks above the bar graph at "7 DAY" shows the statistical significant level as follows (n=6): * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$)

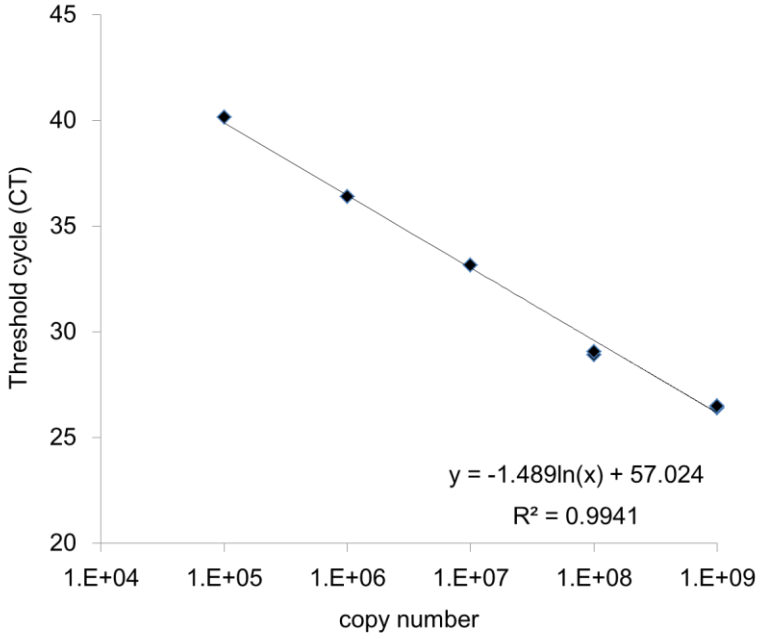


Figure 3-9 The standard line for the real-time PCR using the primer set for *dsrA* gene of Grp3 SRB and the standard DNA templates of the artificial gene of *dsrA*. The data are presented as mean (n=3)

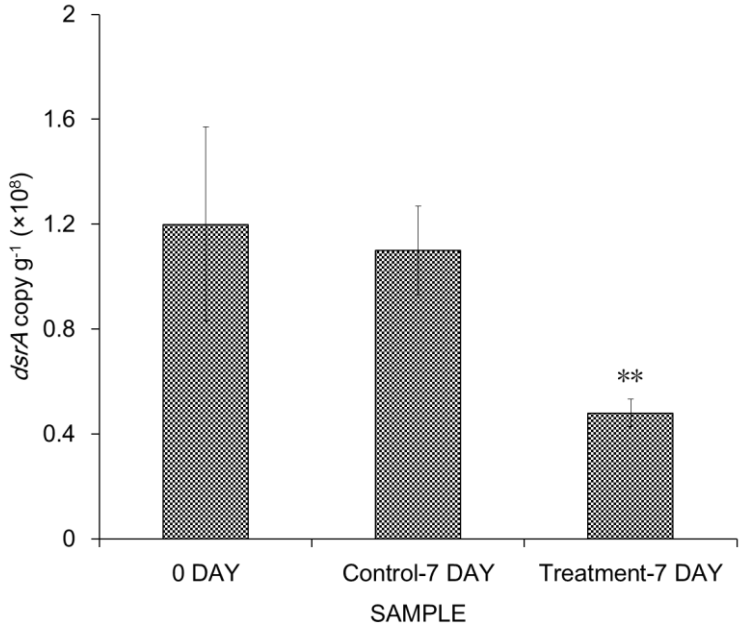


Figure 3-10 Changes of SRB as the copy number of *dsrA* gene in the sediments of each group. Data are presented as mean (error bar = SD). The asterisk marks above the bar graph at "Treatment-7 DAY" shows the statistical significant level as follows (n=9): * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$)

3.4 DISCUSSION

The results of this study clearly indicated that the generation of AVS in the sediment could be inhibited by the addition of $Mg(OH)_2$. The AVS content of the sediment was apparently related to the generation of H_2S under anaerobic condition of the sediment. Kanaya and Kikuchi (2009) had demonstrated that FeS generated by binding H_2S with free Fe^{2+} ion, will not increase when free Fe^{2+} ion doesn't exist, even if numerous H_2S were generated. Thus, the value of AVS is almost consistent with the total value of FeS and H_2S, it is suggested that the fluctuation of H_2S will mainly affect the AVS in case the free Fe^{2+} ion doesn't exist.

When AVS was decreased in the experiment-1, the apparent increment of DOC of the sediment was observed in the treatment group after the 20-day incubation while the increment of DOC in the control group was not statistically significant. An interpretation on this phenomenon was considered on the basis of the character of SRB as follows. SRB can widely utilize different organic compounds such as short chain fatty acids, sugars and amino acids (Rabus *et al.* 2006; Widdel 1988), which are DOC acting as electron donors or carbon sources for dissimilatory sulfate reduction (Hansen 1993; Widdel & Bak 1992). Thus, the decrement of the abundance and/or the activity of SRB lead to the decrease consumption of DOC. Therefore,

DOC could accumulate more in the sediment of the treatment group compared to the control group.

Accordingly, the stronger evidence for the hypothesis was obtained by the direct measurement of H_2S and SRB. In experiment-2, the results showed that the sediment maintained at the alkaline range by adding $Mg(OH)_2$ could inhibit the growth of SRB and H_2S generation. O'Flaherty *et al.* (1998) also observed that when the pH of the culture medium raised to 8, the growth rate of SRB decreased. Additionally, the growth of the SRB was almost stopped when the pH rose above 9. Meanwhile, H_2S content was observed to decrease along with the pH increase. According to these results, the decrease of SRB population, H_2S and AVS content were related to inhibiting SRB activity in the alkalized sediment by spraying $Mg(OH)_2$ to the sediment.

On the other hand, it was an obvious question that the H_2S concentration of the treatment group was higher than that of the control group after only one-day incubation. One of the possible reasons might be related with H_2S electrolytic dissociation in water. The first step dissociation of H_2S is in the equilibrium with HS^- and H^+, where the pKa value is 6.9 at 20˚C (Stumm & Morgan 1995). This equilibrium could move to the HS^- side in the alkali condition, that is, HS^- ion was more than H_2S of volatile form when the sediment maintained at a high pH level such as the initial stage of incubation. Subsequently, SRB activity in control group could recover and generate H_2S when the sediment condition came back to anaerobic condition during the incubation. This explained the real-time PCR of SRB in experiment-2.

Cook *et al*. (2008) achieved to design TaqMan probes with primers to specifically target the *dsrA* gene of Group 1A, Group 1B, Group 2A, and Group 3A slurry SRB and enumerate these organisms by quantitative real-time PCR. They used Grp1 and Grp1B primer sets to target *Desulfobulbus*-like species, Grp2 primer sets were used to target *Desulfovibrio*-like species, and Grp3 primer sets to target *Desulfovibrio sp.*. *Desulfovibrio sp.* in the Grp3 was identified from an inner bay in Japan (Takii *et al.* 2008). Spence *et al.* (2008) also succeeded to develop the real-time PCR of SYBR assay for Group 1, Group 2 and Group 3 of SRB for manure samples. In present study, the primer sets Grp3 was applied to specifically amplify a 119 bp target fragment of *dsrA* gene of SRB. On the other hand, the real-time PCR with the primer sets for Grp1 and Grp2 never showed the normal amplification curves of the samples even though the several PCR conditions were examined. Therefore, a portion of SRB could be only detected and measured in present study. Considering the consistency with the experimental results on the decrement of AVS and H_2S, it is reasonable that the activity of the other major groups of SRB might be inhibited by the addition of $Mg(OH)_2$ as well as Grp3 SRB, though the strength

of the effect for each group of SRB might be different. In the further study, it will be important to investigate the effect of $Mg(OH)_2$ for the whole SRB community as well as other bacterial communities in the sediments because the SRB communities have a significant role in the benthic ecosystem (So & Young 1999; Phelps *et al.* 1998; Zhang & Young 1997).

In conclusion, the addition of $Mg(OH)_2$ raised the pH of the sediment over to 8 which weakened the sulfate-reducing activity of SRB. Hence, the generation of AVS and that of H_2S in sediments were inhibited. Moreover, the increase of the DOC observed in treatment group sediment also strongly indicated the decrease of the activity of SRB, because DOC is a major substrate of SRB. In summary, the usefulness of $Mg(OH)_2$ for the sediment remediation was ensured in this study.

3.5 CONCLUSION

(1) In the Experiment-1, the pH of $Mg(OH)_2$ treatment group was significantly increased from 7.43 to 8.25 after the 20-day incubation; it was higher compared to the control group which pH was maintained around 7.6.

(2) In the Experiment-1, the AVS content of $Mg(OH)_2$ treatment group was significantly reduced and the value was lower than the control group after the 20-day incubation.

(3) In the Experiment-1, the concentration of interstitial water DOC in the treatment group and the control group increased after the incubation from initial value of 15.4 mg L^{-1} to 40.1 mg L^{-1} and 29.0 mg L^{-1} respectively.

(4) In the Experiment-2, simultaneous decrease of H_2S and the *dsrA* gene copy number of Grp3 SRB was observed at the end of the 7-day incubation by adding $Mg(OH)_2$.

(5) It is concluded that the loss of activity of SRB was strongly related to the decrease of sulfide (H_2S and AVS) and the increase of DOC was due to the alkalization of the sediment by adding $Mg(OH)_2$.

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4 THE EFFECTS OF MAGNESIUM HYDROXIDE FOR THE MICROBIAL COMMUNITY IN SEDIMENT OF AN EUTROPHIC CLOSED BAY

4.1 INTRODUCTION

The hypoxic condition of submarine has exacerbated the deoxidization and sulfurization of marine sediments not only in enclosed estuaries and inner bays, but also in coastal fish-farming areas (Hargrave *et al.* 2008). Furthermore, the increase of hydrogen sulfide (H_2S) has inhibited the microbial degradation of protein-rich organic matter (Hoppe *et al.* 1990), and adversely threatened the survival of benthos, fishes and other organisms (Capone & Kiene 1988; Smith *et al.* 1977; Gamenick *et al.* 1996; Vismann 1996; Arndt *et al.* 2013). It also accelerated the deterioration of sediment ecosystem that resulted in a serious commercial loss to coastal fisheries (Devai & De Laune 1995; Avnimelech & Ritvo 2003). Sulfate-reducing bacteria (SRB) are a diverse group of anaerobic microorganisms which are responsible for H_2S production (Stahl *et al.* 2002). Many kinds of SRB have been identified and quantified in many anaerobic marine or estuary sediments (Perez-Jimenez & Kerkhof 2005; Leloup *et al.* 2007). Moreover, based on comparative analysis of 16S rRNA gene sequences, SRB have been grouped into 7 known phylogenetic lineages, and most of the SRB were classified in 23 genera within the Deltaproteobacteria, followed by gram-positive SRB within the Clostridia (Muyzer & Stams 2008).

Magnesium hydroxide ($Mg(OH)_2$) is a sediment improving agent that had been widely applied in cage aquaculture areas for sediment remediation. Xia *et al.* (2017) have performed an incubation experiments and demonstrated the reduction of SRB by adding $Mg(OH)_2$ through real-time PCR method where the decrease in H_2S was observed, indicated that the loss of activity of SRB were strongly related to the decrease of sulfide (H_2S and AVS) by $Mg(OH)_2$ addition.

These researches mentioned above primarily focused on the relationship between the reduction of H_2S generation and the suppression of SRB growth. However, it is significant to comprehend that the changes of microbial community in the sediment by the addition of $Mg(OH)_2$ was because SRB exist in the community with the complex relationships to other microbes in the sediment. Hence, this chapter was done to examine the effects of $Mg(OH)_2$ for the reduction of sulfide in the sediment by a laboratory experiment. Secondly, the next-generation sequencing (NGS) method (Sogin *et al.* 2006) for analyzing the changes of the

microbial communities through spraying $Mg(OH)_2$ was also applied.

4.2 MATERIALS AND METHODS

4.2.1 Study Site and Sample Collection

Omura Bay is a typical eutrophic inner bay located in the center of Nagasaki Prefecture (32° 57′ 0″ N, 129° 52′ 30″ E), Japan. The sediment samples were collected from the Togitsu Port Ferry Terminal (32° 50' 0″ N, 129° 50' 0″ E), which located in the southeast water area of Omura Bay. The collection of samples was carried out on August 11, 2015. All sediment samples were stored in cooler boxes and were quickly transported back to the laboratory within 2 hours. After removing the gravel and pebbles, fine sediment (< 2 mm grain size) were frozen (-20°C) for further analysis and DNA extraction.

4.2.2 Experimental Setup

The collected sediment samples were thoroughly mixed after removing visible small litters such as pebbles, shells, small benthic animals and plant debris. A total of 170g of sediments were put in a small plastic container (width is 3.5 cm, depth is 3.5 cm, length is 7 cm). For the treatment group, clear water (Ube Materials Co., Yamaguchi, Japan) which contained 2mm diameter $Mg(OH)_2$ granules was mixed thoroughly with the sediments (Figure 4-1). Meanwhile, there was no $Mg(OH)_2$ added on the control group. Three sediment containers were prepared for each group (three replicates).

All plastic containers, both control group and treatment group, were placed in a rectangular closed plastic reactor (45 cm in length, 30 cm in width and 35 cm in depth) separately. Each reactor was filled with 20L seawater collected from Omura bay, and aerated with nitrogen gas at 100 mL min^{-1}. The sea water in each reactor was kept at 26ºC using AQUA COOLER SLIM202 constant-temperature water circulator (MARUKAN, NISSO Department, Osaka, Japan) during the incubation which lasted for ten (10) days (Figure 4-2a & b). The sediments from each group were collected after the incubation and were undergone analysis. The original sediment before the incubation was also analyzed.

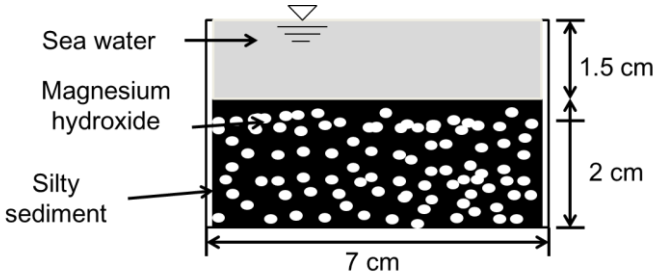

Figure 4-1 Magnesium hydroxide ($Mg(OH)_2$) mixed with silty sediments

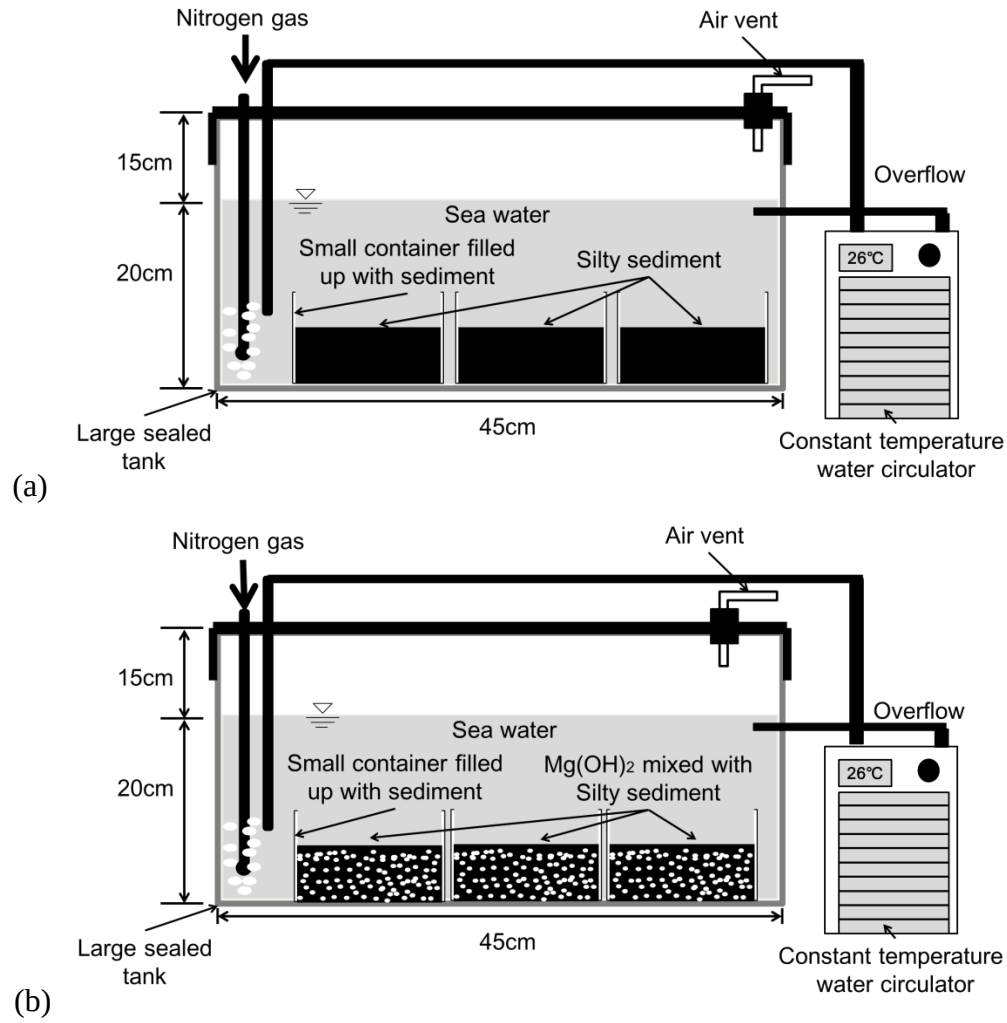

Figure 4-2 Schematic figure of the equipment for Experiment-1. (a) Control group without $Mg(OH)_2$, (b) Treatment group with $Mg(OH)_2$ granules scattered on the sediments

4.2.3 Chemical Parameters of the Sediments

The water content of the sediment was measured before the incubation. Five parallel sediment samples in each container were measured every 4 hours at 105ºC using a laboratory oven (MOV-

212F-PE Panasonic, Osaka, Japan). The values were calculated as follows: [(weight of fresh sediment) − (weight of dried sediment)] / (weight of fresh sediment) (Xia *et al.* 2013). The dissolved oxygen (DO) and the pH of the sediment were measured using the same method that was done with Experiment-2 in the previous study (Xia *et al.* 2017).

Dissolved organic carbon (DOC) in interstitial water was measured using NPOC (Non-Purgeable Organic Carbon) measurement method in accordance with the standard measurement methods (JIS K0101:1998 Testing Methods for Industrial Water) by using TOC-L equipment (Shimadzu, Co., Kyoto, Japan). The interstitial water samples were injected into the quartz reaction tube and were treated with 20% phosphoric acid, sparged with CO_2-free air at 680ºC for 90 seconds to remove inorganic carbon (Sugimura & Suzuki 1988). The automatically averaged value of DOC in three replicates was used. The acid volatile sulfides (AVS) in the sediment samples were measured in a sulfide detection tube (Detector Tube No. 201H; GASTEC, Kanagawa, Japan), which was used as a surrogate parameter of TS (Rickard & Morse 2005). Five repeating measurements for each sample were carried out. The pH, DOC and AVS of all the sediment samples were measured before and after the 10th day of incubation.

4.2.4 Molecular Biological analysis of the Microbial Community

4.2.4.1 *DNA Extraction*

The sediment samples of three containers from control and treatment groups were mixed into one subsample. Total genomic DNAs were extracted from each subsample after the 10^{th} day of incubation with the FastDNA Spin Kit for Soil (MP Bio Japan, Tokyo, Japan) following the instruction of manufacturer. The total genomic DNAs from original sediments were also extracted. Extracted DNA samples were eluted into TE Buffer (Tris-EDTA Buffer, pH 8.0) to a final volume of 50 μL and stored at -20 °C until use.

4.2.4.2 *Quantitative real-time PCR of Bacterial 16S rRNA gene*

The copy number of bacterial 16S rRNA gene was quantified in each extracted DNA sample using real-time PCR TaqMan probe method. Amplification for each DNA samples was carried out in triplicate. The forward primer BACT1369F (5'- CGG TGA ATA CGT TCY CGG -3'), reverse primer PROK1541R (5'- AAG GAG GTG ATC CRG CCG CA -3'), and the TaqMan probe TM1389F (5'- FAM-CTT GTA CAC ACC GCC CGT C-BHQ-1 -3') (FAM=5-carboxyfluorescein, BHQ=black hole quencher 1) were used to detect the target region of bacterial 16S rRNA gene (Suzuki *et al.* 2000). The genomic DNA of cyanobacteria *Microcystis* sp. strain NIES843 was used as external standard to determine the copy number of 16S rRNA

gene. The thermal cycling condition consisted of an initial denaturation step of 95°C for 30s, followed by 45 cycles each subjected to a denaturation step of 95°C for 10s, as well as the annealing and extension step of 60°C for 30s.

4.2.4.3 *Next-Generation Sequencing (NGS)*

PCR amplification of the extracted DNA from each sediment sample was performed using a barcode tagged primer set for pyrosequencing of the bacterial 16S rRNA genes. This primer set targeted the V4 hyper-variable region (Zheng *et al.* 2016) of the 16S rRNA genes using the 515F (5'- GTG YCA GCM GCC GCG GTA A -3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT -3') primer set (Caporaso *et al.* 2011; Hu *et al.* 2014). The PCR was performed at ABI Veriti Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) with GoTaq® DNA Polymerase (Promega Corporation, Madison, USA). A total of 20 µL PCR reaction system comprised 10µL 2× GoTaq® Green Master Mix Solution (Promega Corporation, Madison, USA), 0.4 µM each of forward and reverse primers (final concentration), and ca. 10 ng of sediment DNA was used as a template. Nuclease-Free Water was added to make a final volume of 20 µL. Each library was amplified using the following protocol: for the initial denaturation step of 94 °C for 5 min, followed by 30 cycles each subjected to a denaturation step of 94 °C for 30s, annealing step of 54 °C for 30s and extension step of 72 °C for 40s, and a final elongation step of 72 °C for 5 min. All the amplifications were checked using electrophoresis with 1.5% agarose gels. The bands were extracted and purified with the QIAquik Gel Extraction Kit (QIAGEN (corporate), Venlo, Netherland). DNA was quantified using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and was then mixed in equivalent proportions. The sequencing of the amplifications was performed on Roche GS Junior pyrosequencing machine (Roche Diagnostics Corporation, Branford, CT, USA) according to the manufacturer's instructions.

Bacterial 16S rRNA gene of the original sediments and that of each sediment sample in control and treatment groups after the 10-day incubation was analyzed using Ribosomal Database Project (RDP) pipeline for the investigation of the bacterial community structure. The taxonomic composition was determined using the "Classifier" tool in RDP pipeline. The sequences of the bacterial 16S rRNA gene were aligned using the "Aligner" tool and the aligned data were clustered by using the "Complete linkage clustering" tool in RDP pipeline. The species clusters were defined using the 97% similarity cutoff of the bacterial 16S rRNA gene sequences. The abundance (copy number) of each taxonomic cluster was calculated by multiplying the relative abundance of sequence reads of each taxonomic cluster to the copy number of total bacterial 16S

rRNA gene obtained from the quantitative real-time PCR analysis.

4.2.5 Statistical Analysis

The data were expressed as mean ± standard deviation (SD) and all statistical analyses were carried out in R software (version 3.3.1) (The R Foundation for Statistical Computing, Vienna, Austria). The significant difference (P<0.05 was considered significant) in all sediment samples before and after the 10-day incubation was determined using a student t-test; the Welch's method (Welch 1951) was adopted to compare two objects which had unequal variance assumption (Moder 2010). Redundancy Analysis (RDA) (based on the sequence reads of dominant genera) was performed using the "vegan" package of R, in order to illuminate the relationship among the changes of chemical parameters of sediments by spraying $Mg(OH)_2$, as well as, the bacterial community composition in the sediments.

4.3 RESULTS

4.3.1 Effects of $Mg(OH)_2$ on pH, AVS and DOC of Sediments

The DO concentrations of seawater in two reactors were stable around 0.4 mg L^{-1} during 10 days of incubation. The water content of all sediment samples ranged from 56.5% to 61.9%.

Chemical parameters of sediments were measured before and after the 10-day incubation, and the results were shown in Figure 4-3. The pH initial value was 7.19 ±0.05, and the AVS content of original sediment was at 1.28 ±0.12 mg dry-g^{-1}. The DOC content of interstitial water in the original sediment was 125.3 ±0.80 mg L^{-1}. After the 10-day incubation, the pH of the sediments in the $Mg(OH)_2$ treatment group have significantly increased to 8.68 ±0.11 (P=0.0011), while the AVS have significantly decreased to 1.11 ±0.07 mg dry-g^{-1} (P=0.0002). The DOC content of the treatment group (183.9 ±5.65 mg L^{-1}) and control group (136.5 ±6.33 mg L^{-1}) both significantly increased from the initial DOC (P<0.001 and P=0.013, respectively). Furthermore, the DOC of treatment group after the 10 days incubation was higher than that of the control group (P<0.001).

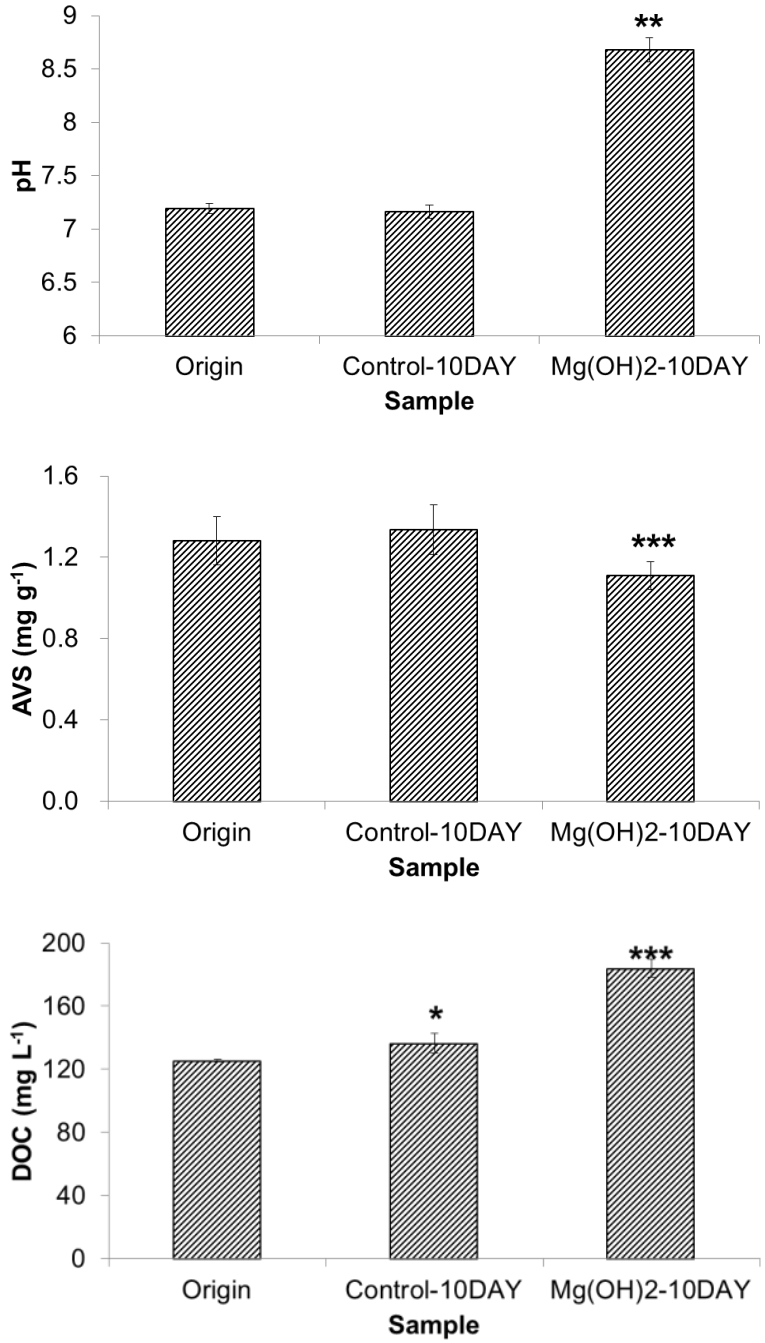

Figure 4-3 Changes of pH, AVS and DOC of sediments before and after the incubation; Statistical significant level as follows: * P<0.05, ** <0.01, *** P<0.001

4.3.2 Effects of $Mg(OH)_2$ on Bacterial Community

A total of 26561 effective sequences of 16S rRNA gene from all the sediment samples were identified and used for the analyses of abundance, diversity and the taxonomic comparison of microbial community. At 97% gene similarity, 8947 sequence reads were classified into 2396 OTUs in original sediments. After the incubation, 6148 sequence reads were classified into 1803

OTUs in control group and 11466 sequence reads were classified into 2318 OTUs in treatment group sediments. Rarefaction curves were generated by plotting the number of sequences against the number of OTUs observed at the 97% similarity level. The results showed that all curves were unsaturated, and the increase rate of OTUs against the sequences in treatment group was at the lowest (Figure 4-4).

The bacterial community composition before and after the incubation were analyzed at phylum and genus levels. A total of 24 phyla of bacteria were represented amongst the classified sequences, and the top 10 phyla were selected as the dominant phyla. The most major phylum was classified to Proteobacteria, the Deltaproteobacteria and Gammaproteobacteria class were dominant within the phylum, which accounting 36.26-45.85% of the total sequences detected in all sediment samples (Figure 4-5). Firmicutes was identified as the second major phylum in this study, it covered 18.62% of the total sequence reads detected in treatment group sediments. Chloroflexi and Acidobacteria were also showed higher dominance in treatment group sediments, while Bacteroidetes were much more dominant in original sediments.

Using the real-time PCR, the 16S rRNA gene of total bacteria in the sediments was quantified. The copy number of 16S rRNA gene detected in original sediments was $2.65 \pm 0.06 \times 10^{10}$ copy g^{-1}. After the incubation, these increased to $3.93 \pm 0.37 \times 10^{10}$ copy g^{-1} in the control group, and decreased to $1.01 \pm 0.07 \times 10^{10}$ copy g^{-1} in the treatment group. Based on the copy numbers of 16S rRNA gene of total bacteria and the relative abundance of the classified bacterial genera by NGS analysis, the copy number of 16S rRNA gene in each dominant genus was calculated (Figure 4-6).

In Figure 4-6, the significant decrease of SRB was evidently showed; *Desulfobulbus, Desulfopila* and *Desulfsarcina* belong to Delta-proteobacteria in the treatment group sediments after the incubation. Meanwhile, *Haliea* and *Thioprofundum* that belong to Gamma-proteobacteria, and *Acidobacteria* Gp10 and *Acidobacteria* Gp23 that belong to Acidobacteria have decreased in the treatment group. However, these genera mentioned above have increased in the control group sediments. On the contrary, *Fusibacter, Alkaliphilus* and *Tindallia* that belong to Firmicutes were significantly increased in the treatment group sediments after the incubation.

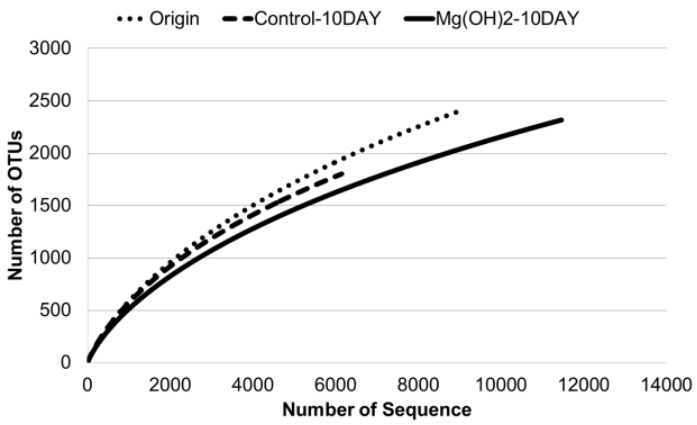

Figure 4-4 Rarefaction analysis of bacterial 16S rRNA gene from all sediment samples.

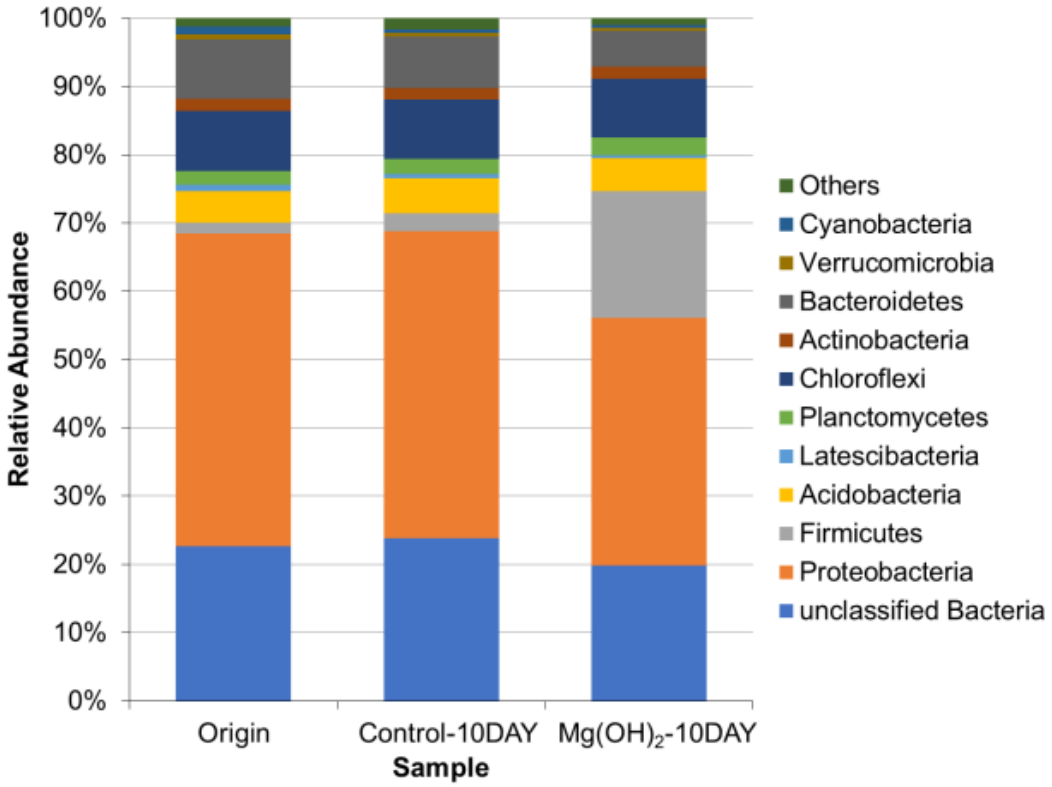

Figure 4-5 Bacterial composition in the sediment samples at the dominant bacterial phyla level. Origin: initial sediment samples; Control-10DAY and $Mg(OH)_2$-10day: sediment samples of control and treatment group after the 10-day incubation.

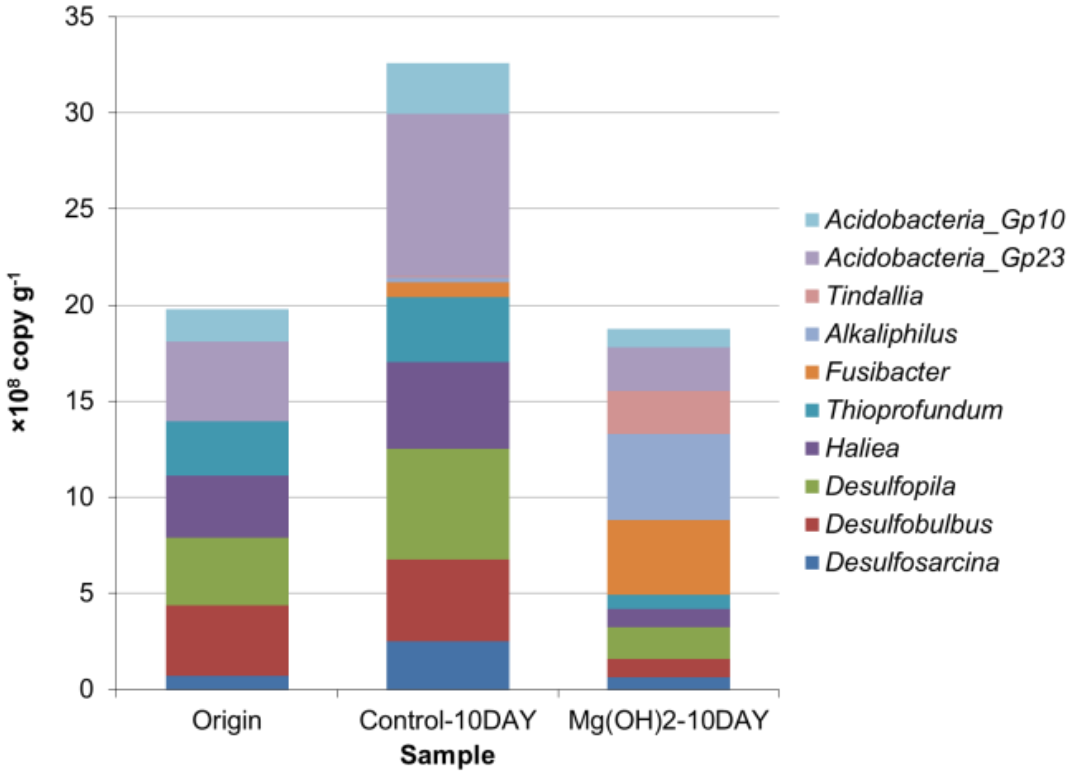

Figure 4-6 Bacterial composition in the sediments at the dominant bacterial genera level. Y axis is the copy number of bacterial 16S rRNA gene of dominant genera. Origin: initial sediment samples; Control-10DAY and $Mg(OH)_2$-10DAY: sediment samples of control and treatment group after the 10-day incubation.

4.3.3 Relation among pH, AVS, DOC and Bacterial Communities

The pH, AVS, DOC and each copy numbers 16S rRNA gene of bacterial species were used to conduct the redundancy analysis (RDA) to illuminate the relation among them. In Figure 4-7, the first two axes (RDA1 and RDA2) by three parameters (pH, AVS, DOC) explained the 88.44% and 11.55% variation of the bacterial communities, respectively. AVS showed the positive correlation (r=0.954, *P*=0.011) with the RDA1 axis, while pH and DOC negatively correlated with the RDA1 axis (r=-0.966, *P*=0.004 and r=-0.890, *P*=0.001), respectively. The AVS has been gradually increasing whereas the pH and DOC has been gradually decreasing from left to right in RDA1 axis.

The dominant genera *Desulfobulbus*, *Desulfopila*, *Haliea* and *Thioprofundum* have showed a significant positive correlation with AVS (*P*<0.001) while it showed a significant negative correlation with the pH (*P*<0.001). On the other hand, *Fusibacter*, *Alkaliphilus* and *Tindallia* have a positive correlation with the pH (*P*<0.001) and a negative correlation with the AVS (*P*<0.001). This showed a clear distinction between the characteristic of the other bacteria (Fig.5).

Moreover, the genera *Fusibacter*, *Alkaliphilus* and *Tindallia* only dominated the treatment group sediments rather than in original sediments (Fig.4). However, only *Natronincola* showed a negative correlation with pH (r=-0.042, *P*=0.914) and AVS (r=-0.008, *P*=0.984) simultaneously, varied from other genera within Firmicutes. The genera Acidobacteria *Gp23* and *Gp10* were also have a negative correlation with pH (*P*<0.05) and a positive correlation with AVS (*P*<0.05).

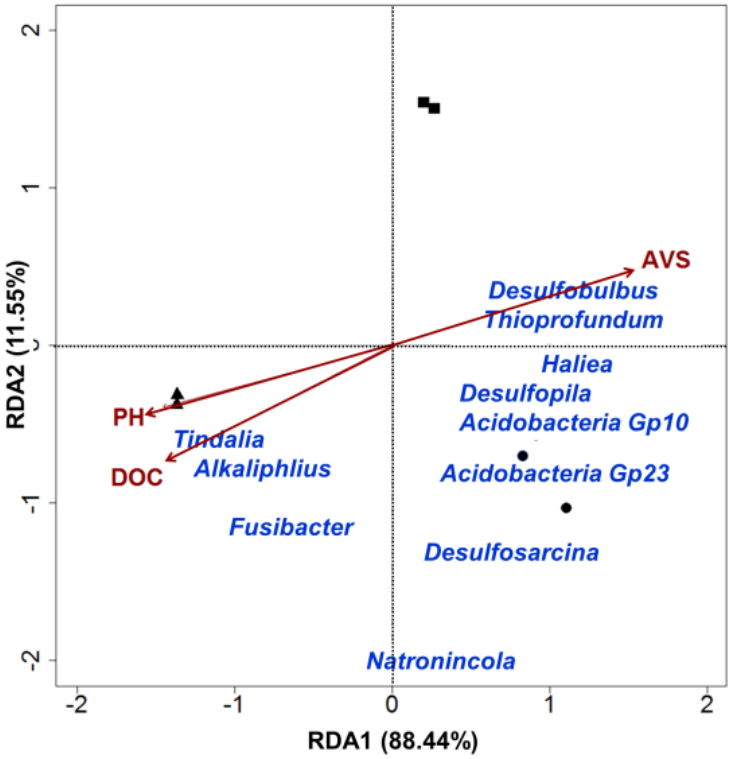

Figure 4-7 Redundancy analysis (RDA) plots of the relationship between chemical parameters and bacterial community in the sediment. Red arrows, physicochemical variables; Blue letters, dominant genera; ■, Origin; ●, Control-10DAY; ▲, $Mg(OH)_2$-10DAY.

4.4 DISCUSSION

This study clearly demonstrated that spraying $Mg(OH)_2$ on the sediments not only inhibited the generation of sulfide in the sediment, but also altered the composition of bacterial communities in the sediments. These results were consistent with previous researches as mentioned in the introduction (Zhang *et al.* 2008; Yoshida & Nishino 2005).

Xiong *et al.* (2012) demonstrated that pH was the dominant factor influencing the alkaline sediment community structure, the phylotype richness and the phylogenetic diversity. The results showed that the relative abundance of Delta- and Gamma-proteobacteria have lessen while abundance of Firmicutes have surged as the pH risen. Moreover, some ions correlated with pH, such as Mg^{2+}, showed a significant positive correlation with the relative abundance of Firmicutes

(Xiong *et al.* 2012). Smilar results were obtained in treatment group sediments after the incubation due to the alkalization by spraying $Mg(OH)_2$. The relative abundance of Proteobacteria in the treatment group has lessened except Firmicutes which abundance has risen. This showed the difference of bacterial composition with original sediments as presented in Figure 4-5. As shown in Figure 4-6, the reason of the Firmicutes increase was primarily due to the genera *Fusibacter*, *Alkaliphilus* and *Tindallia,* which are alkaliphilic bacteria. Moreover, the RDA analysis clearly demonstrated that these genera have multiplied as the pH increased (Figure 4-7).

The genera *Desulfobulbus* and *Desulfopila* showed the same increasing tendency with AVS along the RDA1 axis. This positive correlation between SRB and AVS was due to the reduction of sulfate to sulfide through SRB. The genus *Thioprofundum,* which is a chemolithoautotrophic sulfur-oxidizing bacteria (SOB) belongs to Gamma-proteobacteria, has also showed a positive correlation with AVS in RDA plot. This result indicated that SRB and SOB identified in this study have existed symbiotically in the sediments. *Thioprofundum* can use oxygen or nitrate as an electron acceptor, and the reduced inorganic forms of sulfur (such as Thiosulfate ($S_2O_3^{2-}$), elemental sulfur (S^0) and tetrathionate ($S_4O_6^{2-}$)) as electron donors for their chemolithotrophic growth. Moreover, this genus of bacteria uses carbon dioxide (CO_2) as a sole carbon source (Mori *et al.* 2011). In this experiment setup, the DO content of seawater above the surface of the sediments was kept around 0.4 mg L^{-1}, that could supply the oxygen for SOB to maintain the respiration activity. On the other hand, the oxidized forms of sulfur produced by SOB, such as sulfate, could be used by SRB as an electron acceptor (Dubilier *et al.* 2001). The electron donors of SRB such as dissolved organic carbon (DOC) and hydrogen (H_2) were taken up from sediments. Furthermore, products of fermentation such as organic acids from the sediments that accumulated during anaerobic metabolism would provide the SRB with an ideal energy source. Van den Ende *et al.* (1997) have demonstrated that the cycling of oxidized and reduced sulfur compounds between the sulfate-reducing and sulfide-oxidizing symbionts were the major factor for continuous cultures with free-living SRB and SOB.

In previous study (Xia *et al.* 2017), the significant increase of DOC content in $Mg(OH)_2$ addition treatment group after a 20-day incubation was attributed to the decrease in the abundance and activity of SRB. In this study, a similar result was observed in which the DOC content after the 10-day incubation in the treatment group sediments was higher. Meanwhile, the population of Firmicutes (the genus *Fusibacter*, *Alkaliphilus* and *Tindallia*), which are mostly alkaliphilic anaerobes, had significantly increased. These bacteria can produce organic acids with low molecular weights as the major fermentation products (Castaldelli *et al.* 2013). As a result,

abundance decrease and the activity of SRB accelerate the accumulation of DOC in the sediments (Xia *et al.* 2017), also the increase of alkaliphilic anaerobes such as Firmicutes which can produce low molecular weight organic matters, might be another major reason for the increase of DOC observed in the treatment group in this study.

In addition, *Acidobateria* Gp10 and *Acidobacteria* Gp23 have demonstrated that anaerobic bacteria dominantly inhabiting marine sediments with wide range of pH from 6 to 8.5 (Wang *et al.* 2012). They can utilize a variety of sugars, amino acids, alcohols, cellulose and chitin as the carbon sources for fermentation to produce organic acid (Kielak *et al.* 2016). Consequently, the increase of *Acidobacteria* Gp23 observed in the control group contributed to the accumulation of DOC in the sediments (Kielak *et al.* 2016; Ward *et al.* 2009). The observed increment of DOC in control group sediments might be primarily due to the continuous supply of DOC by Acidobacteria, despite the consumption of organic carbon due to the increase of SRB in the sediments.

On the other hand, the rarefaction curves of all sediment samples failed to reach a saturation level as the richness of bacterial species in the sediments collected from Omura bay were comparatively high. In addition, the rarefaction curve of the treatment group was located below than that of the original group, implying it to have a lower bacterial richness compare to the original sediments. This was consistent with the result of Torsvik *et al.* (1998) and Horner-Devine *et al.* (2004), which suggested that the higher nutrient availability leads to a decrease in species diversity. The increased DOC content in the treatment group implied that there is a high carbon or energy resources existed in the sediments. At this high resources availability, the space and resource in the sediments became less patchy and the species diversity was reduced due to the competitively superior dominant species.

The discussion above involved a variety of biological processes demonstrating that the addition of $Mg(OH)_2$ in an anaerobic sediment will make it more microbially diverse. Still, the impact of $Mg(OH)_2$ to the microbial community among sediment remains to be elucidated. Moreover, further study is needed regarding on the interplay between sediment microbial community and its chemical and nutrient characteristics.

4.5 CONCLUSION

(1) After the 10-day incubation, the pH value of the sediments and DOC content of interstitial water in treatment group have increased, while the AVS content have decreased.

(2) The NGS analysis revealed that the changes in the composition of bacterial community. The addition of $Mg(OH)_2$ in sediments showed a decrease in number of Delta-proteobacteria and

increase number of Firmicutes.

(3) The RDA analysis also revealed that SRB within Proteobacteria showed negative correlation (P<0.001) with pH and positive correlation (P<0.001) with AVS. However, Firmicutes has a positive correlation (P<0.001) with pH, and negative correlation (P<0.001) with AVS. Moreover, the genera *Fusibacter*, *Alkaliphilus* and *Tindallia* within Firmicutes have also showed a positive correlation with DOC (P<0.001).

(4) The results of this chapter indicated that adding $Mg(OH)_2$ to the sediment can inhibit the activity of SRB and can reduce the generation of sulfide, and at the same time it can influence the composition of the bacterial communities by accelerating the growth of alkaliphilic bacteria.

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5 THE INFLUENCE OF MAGNESIUM ION AND ALKALIZATION ON GENERATION OF HYDROGEN SULFIDE AND MICROBIAL COMMUNITY IN THE SEDIMENTS

5.1 INTRODUCTION

The previously studies described in chapter 3 and chapter 4 has confirmed that adding magnesium hydroxide ($Mg(OH)_2$) into the sediments to create a weak alkaline environment, it can inhibit the growth of sulfate-reducing bacteria (SRB), meanwhile decrease the generation of hydrogen sulfide (H_2S) and the total sulfide in the sediments, even can influent the dynamics of SRB in the sediments. However, this inhibition effect of ($Mg(OH)_2$) is produced by magnesium ions (Mg^{2+}) or by alkalization is unknown. Moreover, the influence of the changes of pH on the growth and activity of bacterial communities in the sediments is not yet clear.

The importance of Mg^{2+} ions in biological systems has been generally recognized. Reinhart (1988) had summarized that Mg^{2+} is one of the most abundant metal ion in cells, and deeply and intrinsically woven into the cellular metabolism. Mg^{2+} needs bind with specific Mg^{2+} membrane transporters to move Mg^{2+} into the cells. CorA, MgtE and MgtA are three distinct classes of Mg^{2+} transporters identified in gram-negative bacteria *Salmonella enterica* serovar Typhimurium (Hmiel *et al.* 1986, 1989 & Smith *et al.* 1995). Adenosine triphosphate (ATP) must be bound to Mg^{2+} to be biologically active, moreover, Mg^{2+} plays a key role in the stability of all polyphosphate compounds in cells, including those associate with the synthesis of DNA and RNA (Cowan 1995). That is, Mg^{2+} might affect the synthesis of bacterial cells in the sediments.

In this study, laboratory experiments were carried out to evaluate the effect of Mg^{2+} ions and alkalization on the generation of H_2S and the composition of bacterial communities in the sediments.

5.2 MATERIALS AND METHODS

5.2.1 Study Sites and Sample Collection

The study sites were the same with that described in the section 4.2.1. Sample collection was carried out on November 12, 2015. All sediment samples were stored in cooler boxes and were quickly transported back to the laboratory within 2 hours. After removing the gravel and pebbles, fine sediments (< 2 mm grain size) were frozen (-20°C) for further analysis and DNA extraction.

5.2.2 Experimental Setup

The experiment system in this study was similar with the section 3.2.2.2. The polyvinyl chloride (PVC) columns with a polytetrafluoroethylene (PTFE) hollow-fiber microfiltration (MF) membrane (Sumitomo Electric Industries, Osaka, Japan), which crossed the inside of the column were prepared for the sediment column experiment shown in Figure 5-1. Three treatment groups including the $Mg(OH)_2$ addition group, the sodium hydroxide (NaOH) addition group and the magnesium chloride ($MgCl_2$) addition group were prepared. No addition group was also prepared as the control group. Duplicate columns were set for each group.

The sediments were uniformly mixed and then 167 g of sediments were provided to each column of each group. The moisture content of the sediments was measured before starting the incubation, and the mean of 62.9% was used to calculate the water content in the sediments. (1) The $Mg(OH)_2$ addition group was prepared firstly. Four gram of $Mg(OH)_2$ (Ube Materials Co., Yamaguchi, Japan) per column (4 g-$Mg(OH)_2$ /column), which was equivalent to the addition amount of field tests (1 kg/m^2), was mixed well with the sediments, and then poured them into the column and measured the pH of the sediments. The mean of pH in two columns of $Mg(OH)_2$ addition group was 8.28 ± 0.25 (± standard deviation), which showed a weak alkali condition. (2) Based on the pH of $Mg(OH)_2$ addition group, 0.15 g of NaOH (Wako Pure Chemical Industries, Ltd., JIS Special Grade) per column (0.15 g-NaOH /column) was mixed well with the sediments and then poured them into the columns and used them as NaOH addition group. The

mean of pH of two columns in NaOH addition group was 8.60 ± 0.00, which also showed a similar weak alkali condition with the $Mg(OH)_2$ addition group. (3) The magnesium ion (Mg^{2+}) content under the 1 kg m^{-2} addition of $Mg(OH)_2$ was 100 mg L^{-1}, which measured by Chugai Technos Co. (Hiroshima, Japan) using the method of Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). In the $MgCl_2$ addition group, 0.30 g of $MgCl_2 \cdot 6H_2O$ (Wako Pure Chemical Industries, Ltd., JIS Special Grade) per column (0.30 g-$MgCl_2 \cdot 6H_2O$ /column) was mixed with 167 g of the sediments and the Mg^{2+} content was around 338 mg L^{-1} by calculation (Table 5-1).

The columns were filled with seawater collected from Omura bay. Pure nitrogen gas was aerated (100 mL min^{-1}) into each column during the 14-day incubation to prevent the increase of dissolved oxygen (DO) content in the seawater. The columns were incubated in a water chamber and kept at 26ºC using the AQUA COOLER SLIM202 constant-temperature water circulator (MARUKAN, NISSO Department, Osaka, Japan). The syringes (Terumo Co., Tokyo, Japan) were connected to the hollow fiber membranes to collect the interstitial water, meanwhile prevent the surrounding seawater to invade into the sediment column during the incubation (Figure 5-1).

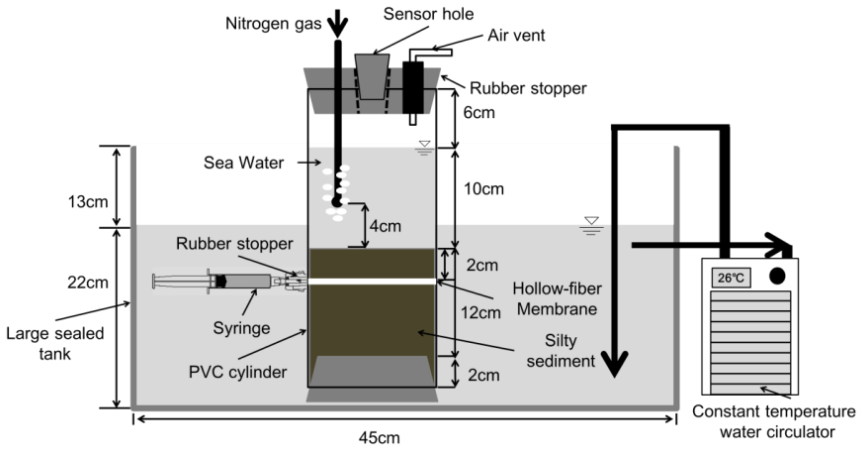

Figure 5-1 Schematic figure of the equipment for incubation experiment. Control group (no addition group) and $Mg(OH)_2$, NaOH, $MgCl_2$ addition groups were set up. Chemical reagent ($Mg(OH)_2$, NaOH and $MgCl_2 \cdot 6H_2O$) were mixed well with sediments before transferred into

each column. Duplicate columns were prepared for each group.

5.2.3 Chemical Parameters of the Sediments

5.2.3.1 pH and Oxidation-Reduction Potential (ORP) of the Sediments, Dissolved Oxygen (DO) of Overlaying Seawater

The dissolved oxygen (DO) of the overlaying seawater in each column was measured by YSI ProODO Optical Dissolved Oxygen Instrument (YSI Incorporated, Ohio, USA). The pH of the sediments was measured at 1 cm depth below the sediment surface in each column by using the pH Electrode, ELP-038 with 3 mm diameter pH sensitive glass tips (TOA DKK, Tokyo, Japan). The oxidation-reduction potential ORP was measured by using a fine platinum electrode with a 0.3 mm tip and saturated KCl, Ag-AgCl reference electrode at 1cm depth below the sediment surface. For each measurement of DO, pH and ORP in each group, one column only measured single time.

5.2.3.2 *Hydrogen Sulfide (H_2S)*

Four (4) mL of interstitial water from sediment was gently collected by the syringe, and then the interstitial water H_2S was quantified by methylene blue method (Yano 2002), which was the same with previously described in section 3.2.3.3.

5.2.4 Molecular Biological analysis of the Microbial Community

5.2.4.1 *DNA Extraction*

The uniformly mixed sediments of each group was divided into two subsamples each weighing ca. 0.5g (wet weight). Microbial DNA was extracted from each subsample before and after the 14-day incubation (Alain *et al.* 2011). The extraction was carried out using the Genomic DNA from soil NucleoSpin® Soil (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and followed the manufacturer's instructions.

5.2.4.2 *Quantitative real-time PCR of Bacterial 16S rRNA gene*

The copy number of bacterial 16S rRNA gene was quantified in each extracted DNA sample using real-time PCR TaqMan probe method, which was the same with the previously described in section 4.2.4.2.

5.2.4.3 *Quantitative real-time PCR of dsrA gene of SRB*

The copy number of *dsrA* gene of SRB was quantified in each extracted DNA template using real-time PCR SYBR method, which was the same with the previously described in section 3.2.3.5.

5.2.4.4 *Next-Generation Sequencing (NGS)*

The library preparation work for next generation sequencing was the same with the previously described in the section 4.2.4.3. Sequencing of the amplifications was performed on the Illumine MiSeq System instruments (Illumina Japan, Tokyo, Japan) according to the manufacturer's instructions.

Bacterial 16S rRNA gene of initial sediment samples in control group and those samples in three treatment groups after the 14-day incubation were analyzed for investigating the bacterial community structure. The abundance (copy number) of each taxonomic cluster was calculated by multiplying the relative abundance of sequence reads of each taxonomic cluster to the copy number of total bacterial 16S rRNA gene obtained from the quantitative real-time PCR analysis.

5.2.5 Statistical Analysis

The data were expressed as mean ± standard deviation (SD) and the following statistical analyses were carried out in R software (version 3.3.1) (The R Foundation for Statistical Computing, Vienna, Austria). The statistically significant differences ($P<0.05$ was considered

significant) among the Control, $Mg(OH)_2$, NaOH and $MgCl_2$ groups after the 14-day incubation was determined by the two-side one-way ANOVA, and $P<0.05$ was considered significant. The one-way ANOVA test was adopted the Welch's method (Welch 1951), since it does not require the equal variance assumption between the groups (Moder 2010). The relationship among environmental factors (pH of sediments, H_2S and Mg^{2+} content of interstitial water) and the composition of bacterial community (the dominant bacterial genera) in the sediments was analyzed by a multivariate linear regression model.

Redundancy Analysis (RDA) (based on the copy number of bacterial 16S rRNA gene in each dominant genera) was performed using the "vegan" package of R software, in order to illuminate the relationship among the H_2S content, environmental factors in each group and the bacterial community composition in the sediments.

5.3 RESULTS

5.3.1 The Initial Conditions of the Sediments in Each Group

The initial sediment conditions of each group were showed in Table 5-1. The pH of each group was measured immediately after adding $Mg(OH)_2$, NaOH and $MgCl_2$, respectively. The content of free Mg^{2+} in the interstitial water in the addition group of $Mg(OH)_2$ was estimated as 1400 mg L^{-1} by the ICP-AES analysis. The interstitial water of the control group sediment showed the Mg^{2+} content of 1300 mg L^{-1}, which was slightly higher than the Mg^{2+} content in the typical sea water at the 3.5% salinity is 1290 mg L^{-1} (Turekian 1968). On the other hand, as the Mg^{2+} content in the 0.300 g addition group of $MgCl_2 \cdot 6H_2O$ was 338 mg L^{-1}, the total Mg^{2+} content was calculated as 1638 mg L^{-1}. Therefore, total content of free Mg^{2+} in the interstitial water was 0.0584 mol L^{-1} and 0.0678 mol L^{-1} in $Mg(OH)_2$ and $MgCl_2$ addition group, respectively (Table 5-1). The ionic strength (I) and the osmotic pressure also were calculated depended on the molar concentration and charge of ions in interstitial water of each group. These two parameters are important factors affecting the properties of the biological cells which are live in sea water. The

both factors in each treatment group were changed within 10% comparing to the original seawater of the control group.

Table 5-1 The initial conditions of the sediments in each group

Group	Addition Agent	Amount of Addition (g per column)	[Mg^{2+}] (mg L^{-1})	[Mg^{2+}] (mol L^{-1})	pH	Ionic strength (I) (mol/L)	Osmotic pressure (bar)
CONTROL	No Addition	0.00	1300	5.42E-02	7.64	0.72	28.14
$Mg(OH)_2$	$Mg(OH)_2$	4.00	1400	5.84E-02	8.28	0.74	28.45
NaOH	NaOH	0.15	1300	5.42E-02	8.60	0.76	29.92
$MgCl_2$	$MgCl_2$ $6H_2O$	0.30	1638	6.83E-02	7.69	0.78	29.19

Control: control (no addition) group; $Mg(OH)_2$: $Mg(OH)_2$ addition group; NaOH: NaOH addition group; $MgCl_2$: $MgCl_2$ addition group.

5.3.2 The Changes of Dissolved Oxygen (DO) in the Seawater, pH and Oxidation-Reduction Potential (ORP) in the Sediments

The 14-day incubation was done with aerating nitrogen gas. The DO content was stable around 0.80-0.90 mg L^{-1} in the control group and around 0.60-0.75mg L^{-1} in three addition treatment groups from the third day after starting the incubation (Figure 5-2).

The pH of the sediments in the $Mg(OH)_2$ addition group had a significant increase from 8.10 to 8.83 at the first three days, and then the value of pH were stable around 8.76 ±0.07 until the end of the incubation. After a slight decrease on the first two days of the incubation, the pH of the sediments in the control group and $MgCl_2$ addition group was stable around 7.55 ±0.21 and 7.35 ±0.05 respectively from the third day of the incubation. The pH of one column of control group significantly increased to 8.09 at the ninth day, but then gradually decreased to 7.4 at the fourteenth day of the incubation. While, the decrease of pH in NaOH addition group lasted five days after starting the incubation, and then the value was maintained around 7.94 ±0.05 from the

fifth day of incubation (Figure 5-3).

The ORP measurement was started from the third day of the incubation. The ORP of each group had a tendency to higher (more positive) reduction potential during the first five days of starting measurement, and then a tendency to lower (more negative) reduction potential was observed from the seventh day of the incubation, which showed a reducing condition of the sediments (Figure 5-4).

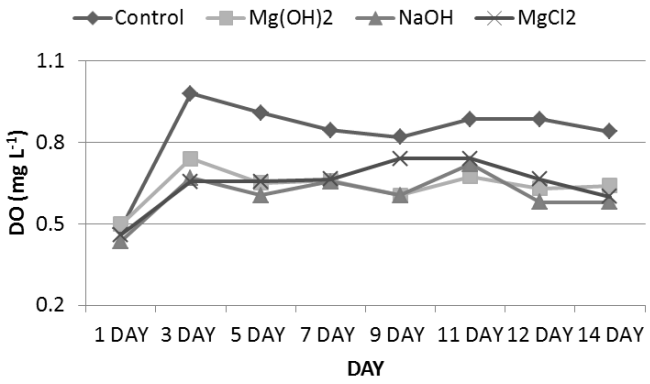

Figure 5-2 Changes of dissolved oxygen (DO) of the overlying seawater in each group. The mean of duplicate columns of data are presented.

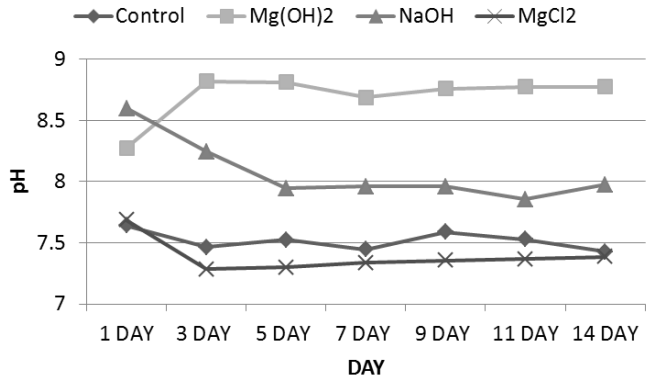

Figure 5-3 Changes of pH of the sediments in each group. The pH was measured at 1 cm depth below the sediment surface. The mean of duplicate columns of data are presented.

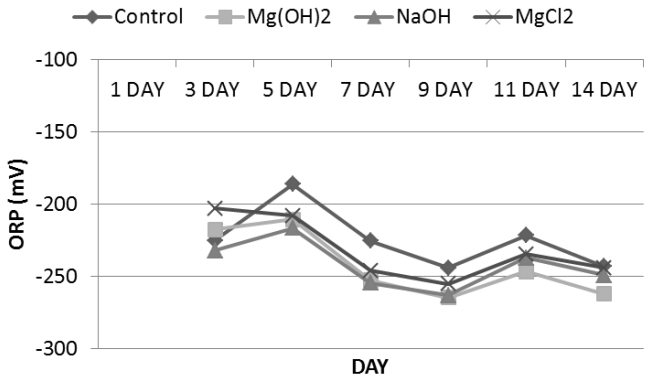

Figure 5-4 Changes of Oxidation-Reduction Potential (ORP) of the sediments in each group. ORP was measured by a fine platinum electrode with 0.3 mm tip and saturated KCl, Ag-AgCl reference electrode. The mean of duplicate columns of data are presented.

5.3.3 The Changes of Hydrogen Sulfide (H_2S) in the Sediments

The H_2S content of the initial sediment samples was 0.829 ±0.04 mg L^{-1}. After the 14-day incubation, the H_2S content of the control group was 0.345 ±0.00 mg L^{-1}. The H_2S content in $Mg(OH)_2$, NaOH and $MgCl_2$ addition group were 0.003 ±0.00 mg L^{-1}, 0.004 ±0.00 mg L^{-1} and 0.003 ±0.00 mg L^{-1} respectively, which were significantly decreased from the control group. Furthermore, compared to the control group, the greatest decrease of H_2S content was observed in $Mg(OH)_2$ addition group (P<0.001) (Figure 5-5).

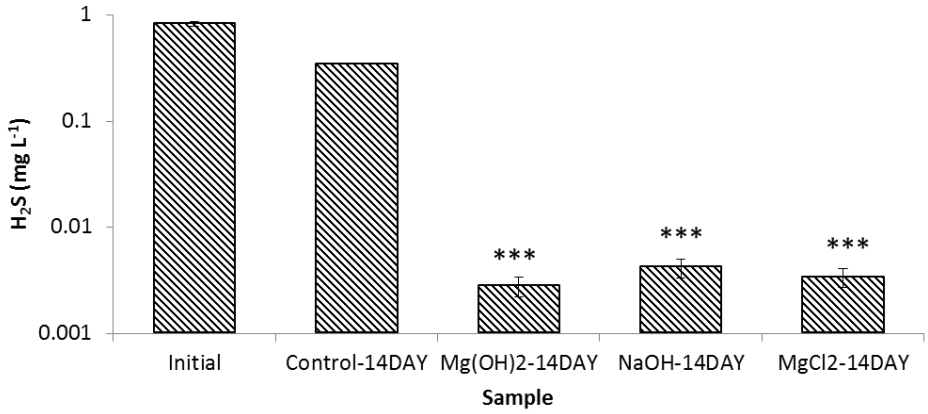

Figure 5-5 Changes of hydrogen sulfides (H_2S) conctent in interstitial water of each group. The

data are presented as mean (error bar = SD) of 6 measurements for initial sediment samples (Initial) and sediment samples of four groups after the 14-day incubation (Control, $Mg(OH)_2$, NaOH and $MgCl_2$-14DAY). The difference between the control group and three addition groups is based on the one-way ANOVA test. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

5.3.4 The Changes of Bacterial Community in the Sediments

5.3.4.1 *Quantification of Sulfate-Reducing Bacteria (SRB) and Total Bacteria in the Sediments*

Using real-time PCR, the *dsrA* gene of sulfate-reducing bacteria (SRB) and the 16S rRNA gene of total bacteria in the sediments of each group were quantified. The standard line (R^2 = 0.9974) for the quantification of *dsrA* gene was illustrated in Figure 5-6. The initial copy number of *dsrA* gene was 2.29 ±1.27 $\times 10^7$ copy mL^{-1}. After the 14-day incubation, the copy number of *dsrA* was decreased to 5.23 ±2.43 $\times 10^6$ copy mL^{-1} in the control group. Compared to the control group, the significant decrease were also observed in three addition groups, which were 1.29 ±0.61 $\times 10^6$ copy mL^{-1} in $Mg(OH)_2$ addition group, 3.98 ±2.34 $\times 10^6$ copy mL^{-1} in NaOH addition group and 1.98 ±1.89 $\times 10^6$ copy mL^{-1} in $MgCl_2$ addition group (Table 5-2; Figure 5-7). In particular, the greatest decrement from the control group was observed in the $Mg(OH)_2$ addition group ($P \leq 0.05$) (Table 5-2).

The standard line (R^2 = 0.9981) for the quantification of bacterial 16S rRNA gene was illustrated in Figure 5-8. The initial copy number of bacterial 16S rRNA gene was 3.63 ±0.918 $\times 10^7$ copy mL^{-1}. After the 14-day incubation, the copy number of bacterial 16S rRNA gene was increased in both control group and NaOH addition group, while an decrease of this gene was observed in both $Mg(OH)_2$ and $MgCl_2$ addition group. Compared to the NaOH addition group, the decrease in $Mg(OH)_2$ and $MgCl_2$ addition group was statistically significant ($P \leq 0.05$) (Table 5-2; Figure 5-9).

Table 5-2 Quantification of *dsrA* gene of SRB and Bacterial 16S rRNA gene of total bacteria in the sediments of each group

Sample	dsrA	STDEV	Bacteria 16S rRNA	STDEV
Initial	2.29E+07	1.27E+07	3.63E+07	9.18E+06
Control-14DAY	5.23E+06	2.43E+06	4.57E+07	2.03E+07
Mg(OH)2-14DAY	1.29E+06	6.19E+05	7.68E+06	1.29E+06
NaOH-14DAY	3.98E+06	2.34E+06	5.44E+07	1.61E+07
MgCl2-14DAY	1.98E+06	1.89E+06	6.22E+06	1.48E+06

Initial: original sediment samples before incubation; Control, $Mg(OH)_2$, NaOH and $MgCl_2$-14DAY: sediment samples of four groups after the 14-day incubation; STDEV: standard deviation.

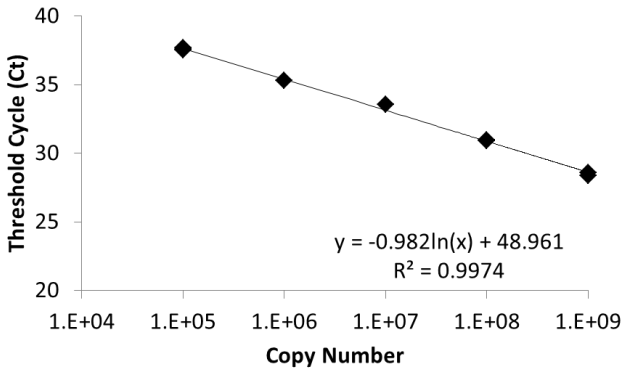

Figure 5-6 The standard line for real-time PCR using the primer set for *dsrA* gene of SRB and the standard DNA templates of the artificial gene of *dsrA*. The data are presented as mean (n = 3).

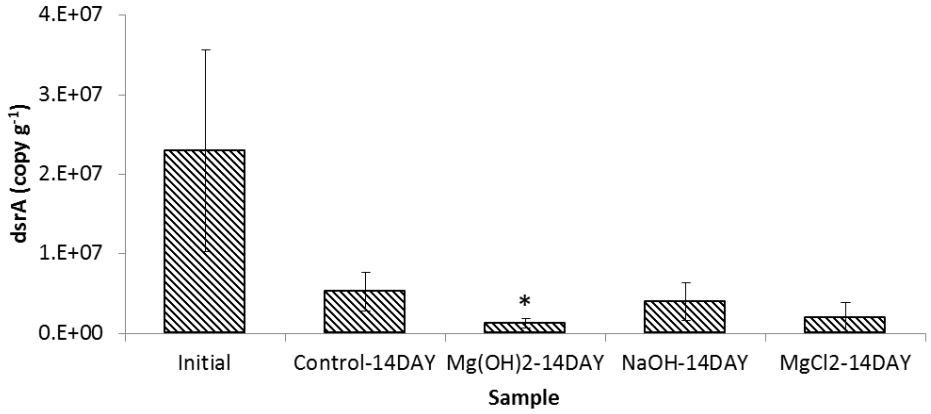

Figure 5-7 Changes of SRB as the copy number of *dsrA* gene in the sediments of each group. Data are presented as mean (error bar = SD) of 12 detections for initial sediment samples (Initial) and sediment samples of four groups after the 14-day incubation (Control, $Mg(OH)_2$, NaOH and $MgCl_2$-14DAY). The difference between the control group and three addition groups is based on the one-way ANOVA test. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

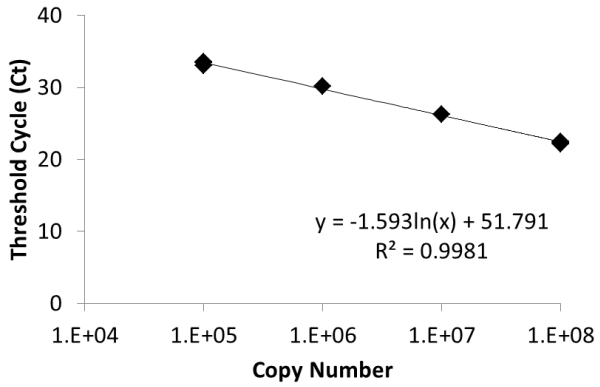

Figure 5-8 The standard line for real-time PCR using the primer set for bacterial 16S rRNA gene and the standard DNA templates of the artificial gene of NIES843. The data are presented as mean (n = 3).

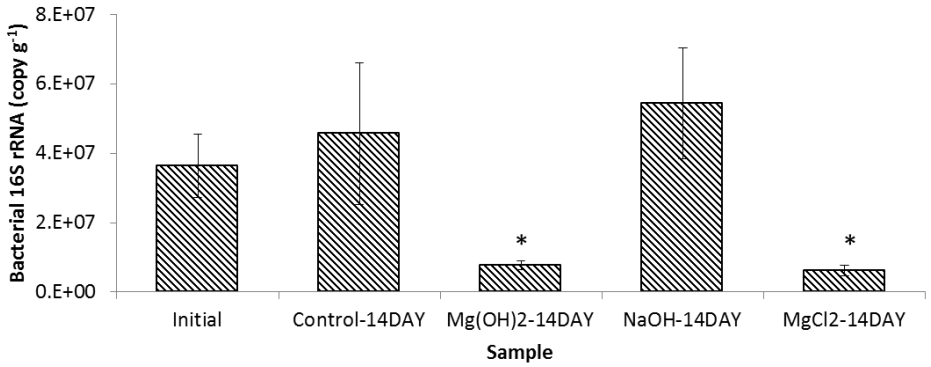

Figure 5-9 Changes of Total bacteria as the copy number of 16S rRNA gene in the sediments of each group. Data are presented as mean (error bar = SD) of 12 detections for initial sediment samples (Initial) and sediment samples of four groups after the 14-day incubation (Control, $Mg(OH)_2$, NaOH and $MgCl_2$-14DAY). The significant difference between NaOH addition group and $Mg(OH)_2$, $MgCl_2$ addition groups is based on the multiple testing. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

The bar graph in Figure 5-10a shows the simple relation between each objective variables (*dsrA* and H_2S) and explanatory variables (Mg^{2+} ions and pH) for each group after 14-day incubation, where Mg^{2+} ions concentration and pH are categorized as follows. "High (high pH)", "Middle (middle pH)" and "Low (low pH)" corresponds to the pH data of all samples from 5th day to 14th day of incubation. "No Mg addition" and "Mg addition" corresponds to the initial sediment conditions of each group before the incubation. Compared with the low Mg^{2+} ions concentration ("No Mg addition" condition), high Mg^{2+} ions concentration ("Mg addition" condition) had a lower copy number of *dsrA* gene and a lower H_2S content. Namely, it was thought that the SRB and the H_2S showed the negative correlations with the Mg^{2+} ions. Contrary to the H_2S content which decreased as the pH level increased, the middle pH level has the highest copy number of *dsrA* gene, while the high pH level showed a similar lower copy number of *dsrA* gene with that at the low pH level (Figure 5-10a).

Furthermore, the relationship between the *dsrA* gene and H_2S is showed by a scatter plot in

Figure 5-10b. The correlation between the *dsrA* gene and the H_2S was unclear though the *dsrA* gene is a chief gene in H_2S generation process by SRB.

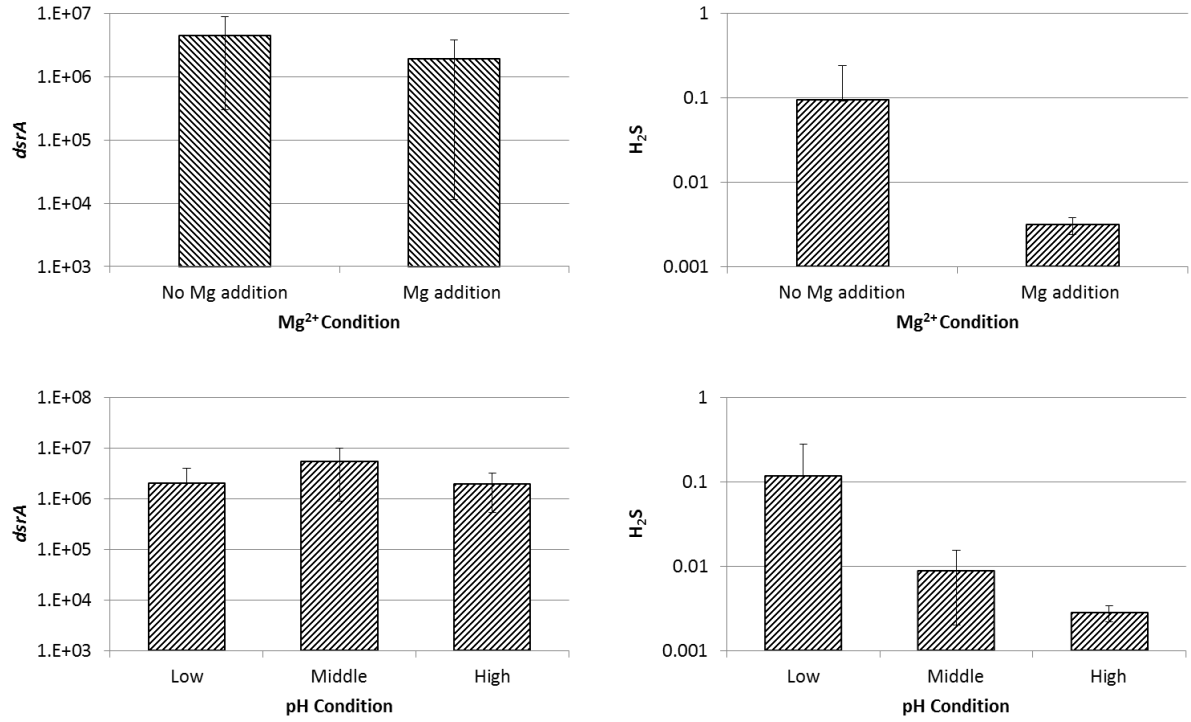

Figure 5-10a Relationship between Mg^{2+} ions / pH and *dsrA* gene of SRB and H_2S. No Mg addition: no addition of compound containing Mg^{2+} ions (Control group and NaOH addition group after the 14-day incubation); Mg addition: addition of compound containing Mg^{2+} ions ($Mg(OH)_2$ and $MgCl_2$ addition group); Low: the mean of pH < 7.5; Middle: 7.5 ≤ the mean of pH < 8.0; High: the mean of pH ≥ 8.0. Each bar of the bar graphs is represented by the mean of *dsrA* gene and H_2S corresponding to each level.

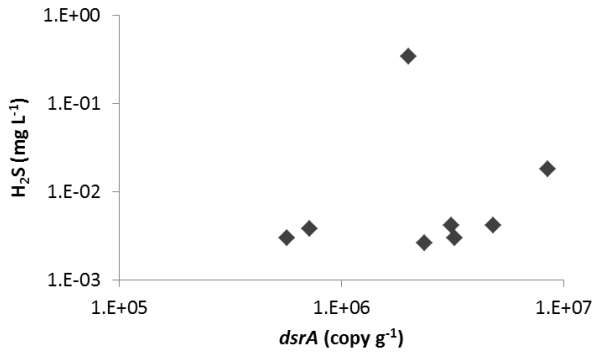

Figure 5-10b Relationship between *dsrA* gene of SRB and H_2S. The scatter plot is displayed by the mean of *dsrA* gene and H_2S corresponding to each group.

Statistical analysis was performed to elucidate the effect of Mg^{2+} ions and pH for SRB (the copy number of *dsrA* gene) and the H_2S generation. A multiple regression (linear model) using two explanatory variables pH and Mg^{2+} ions was applied to summary these effects to SRB and H_2S which were objective variables (Table 5-3). Both the *dsrA* gene of SRB and H_2S have a significant negative correlation with Mg^{2+} ions (the coefficients were -0.27 and the p-value were both less than 0.05) and pH (the coefficients were -0.47 and the p-value were both less than 0.001), and these two factors (Mg^{2+} ions and pH) showed a clear influence to the generation of H_2S than that to *dsrA* gene, because the p-value of F-statistics for H_2S (p=6.89E-07) is smaller than the p-value for *dsrA* gene (p=0.04442). That is, the increase in Mg^{2+} ions and pH separately reduced the growth of SRB and the generation of H_2S. Depending on the results of statistical analysis, the influences of Mg^{2+} ions and pH were likely to reinforce each other to suppress the growth of SRB and the generation of H_2S.

Table 5-3 The results of linear model analysis between objective variables (*dsrA* and H_2S) and explanatory variables (Mg^{2+} ions and pH)

Formula: logdsrA~STDlogMg+STDpH				
Coefficients				
	Estimate	Std. Error	t-value	Pr(>|t|)
Intercept	5.9702	0.1175	50.805	<2E-16***
logMg	-0.2736	0.1277	-2.142	0.0387*
pH	-0.2791	0.1296	-2.153	0.0377*
Residual standard error: 0.7519 on 38 degrees of freedom				
F-statistic: 3.384 on 2 and 38 DF, p-value: 0.04442				
Formula: logH_2S~STDlogMg+STDpH				
Coefficients				
	Estimate	Std. Error	t-value	Pr(>|t|)
Intercept	-2.1856	0.0791	-27.616	<2E-16***
logMg	-0.4686	0.0869	-5.396	3.57E-06***
pH	-0.4656	0.0869	-5.361	3.99E-06***
Residual standard error: 0.5129 on 39 degrees of freedom				
F-statistic: 20.87 on 2 and 39 DF, p-value: 6.891E-07				

The explanatory variable Mg^{2+} concentration was converted to logarithm values, then it was standardized as the mean 0 and the variance 1. The pH was also standardized as the mean 0 and

the variance 1. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

5.3.4.2 The Composition of the Bacteria Community in the Sediments

The bacterial community composition of the initial sediment samples and those after the 14-day incubation were analyzed at phylum and genus levels. A total of 33 phyla of bacteria were identified amongst the classified sequences, and the top 16 phyla, which their relative abundance were more than 1% in all sediment samples during the entire incubation period, were selected as the dominant phyla. The most major phylum was classified to Proteobacteria, especially the class Delta-proteobacteria was dominant within the phylum, which covering 48.8-53.8% of the total sequences detected in all sediment samples during the incubation (Figure 5-11). The phyla Chloroflexi, Acidobacteria and Bacteroidetes were also abundant in all sediment samples. While, the phylum Firmicutes was significantly increased only in $Mg(OH)_2$ addition group after the 14-day incubation, which accounted 12.7% of the total sequence reads detected in the sediments of this group (Figure 5-11).

The dominant genera were determined by the relative abundance of classified phylotypes, which were more than 0.1% in all sediment samples of the initial samples and the four groups during the entire incubation period. Moreover, based on the copy numbers of 16S rRNA gene of total bacteria (Table 5-2) and the relative abundance of the classified bacterial genera by NGS analysis (Figure 5-12), the copy number of 16S rRNA gene in each dominant genus was calculated (Table 5-4).

After the incubation, the greatest decrease of the total amount of bacteria detected in this study was observed in $MgCl_2$ addition group, and followed by $Mg(OH)_2$ addition group (Figure 5-13). The genera *Desulfopila*, *Desulfobulbus* and *Desulfsarcina* belong to the class Delta-proteobacteria were the typical species of sulfate-reducing bacteria (SRB). The significant decrease of the genus *Desulfopila* was observed in $MgCl_2$ addition group after the incubation,

which was decreased from 0.82 ±1.56 $\times 10^6$ copy g^{-1} to 2.41 ±1.61 $\times 10^5$ copy g^{-1}. Contrary to $MgCl_2$ addition group, a significant increase of *Desulfopila* was observed in Control group and NaOH group (Table 5-4; Figure 5-13). The genera *Desulfobulbus* and *Desulfsarcina*, which also belong to the class Delta-proteobacteria, were decreased in $Mg(OH)_2$ and $MgCl_2$ addition groups after the incubation. Especially in $Mg(OH)_2$ addition group, the greatest decrement of these two genera were observed (Table 5-4). The genera *Sulfurovum* and *Sulfurimonas* belong to Epsilon-proteobacteria, *Haliea* and *Thioprofundum* belong to Gamma-proteobacteria and Acidobacteria *Gp10* and *Gp23* belong to Acidobacteria were also decreased both in $Mg(OH)_2$ and $MgCl_2$ addition groups (Table 5-4). The genera *Alkaliphilus* and *Tindallia* belong to Firmicutes were significantly increased in $Mg(OH)_2$ addition group, but were not detected in $MgCl_2$ addition group after the incubation (Table 5-4).

Table 5-4 The copy number of 16S rRNA gene (copy g^{-1}) of top 11 dominant genera in each group.

Phylum	Class	Genus	Initial		Control		$Mg(OH)_2$		NaOH		$MgCl_2$	
			0DAY	STDEV	14DAY	STDEV	14DAY	STDEV	14DAY	STDEV	14DAY	STDEV
Proteobacteria	Delta-proteobacteria	*Desulfopila*	8.20E+05	1.56E+06	1.87E+06	2.41E+06	8.49E+05	6.78E+05	1.45E+06	1.26E+06	2.41E+05	1.61E+05
		Desulfobulbus	2.25E+05	4.25E+05	4.04E+05	3.46E+05	1.41E+05	7.59E+04	4.73E+05	4.15E+05	6.26E+04	3.60E+04
		Desulfosarcina	3.50E+05	6.75E+05	3.96E+05	4.03E+05	5.04E+04	3.85E+04	4.20E+05	3.92E+05	5.30E+04	3.75E+04
	Epsilon-proteobacteria	*Sulfurovum*	1.15E+06	2.23E+06	1.29E+06	1.15E+06	1.89E+05	6.21E+04	1.86E+06	1.91E+06	2.25E+05	1.70E+05
		Sulfurimonas	5.73E+05	1.12E+06	4.28E+05	4.40E+05	1.01E+05	1.36E+04	4.22E+05	4.78E+05	5.47E+04	2.83E+04
	Gamma-proteobacteria	*Haliea*	4.43E+05	8.55E+05	4.94E+05	3.93E+05	5.25E+04	1.06E+04	6.47E+05	6.43E+05	7.66E+04	6.39E+04
		Thioprofundum	2.84E+05	5.52E+05	3.39E+05	4.27E+05	3.24E+04	5.96E+03	3.69E+05	3.97E+05	4.39E+04	3.39E+04
Acidobacteria	Acidobacteria Gp23	*Gp23*	8.61E+05	1.67E+06	9.58E+05	9.61E+05	1.04E+05	2.87E+04	1.45E+06	1.51E+06	1.23E+05	8.25E+04
	Acidobacteria Gp10	*Gp10*	9.48E+05	1.84E+06	7.42E+05	6.03E+05	1.01E+05	5.72E+04	8.18E+05	6.17E+05	1.32E+05	9.92E+04
Firmicutes	Clostridia	*Alkaliphilus*	1.16E+02	2.33E+02	0.00E+00	0.00E+00	5.60E+04	2.99E+04	4.03E+04	6.01E+04	0.00E+00	0.00E+00
		Tindallia	0.00E+00	0.00E+00	6.93E+03	6.62E+03	1.93E+05	5.53E+04	4.43E+04	5.63E+04	0.00E+00	0.00E+00
		Others	1.04E+06	2.00E+06	5.00E+06	1.39E+06	2.12E+06	1.28E+05	3.44E+05	1.60E+06	2.04E+05	1.23E+05

Data are presented as mean of four detections for initial sediment samples (Initial) and sediment samples of Control, $Mg(OH)_2$, NaOH and $MgCl_2$ group after the 14-day incubation (14DAY); STDEV: standard deviation.

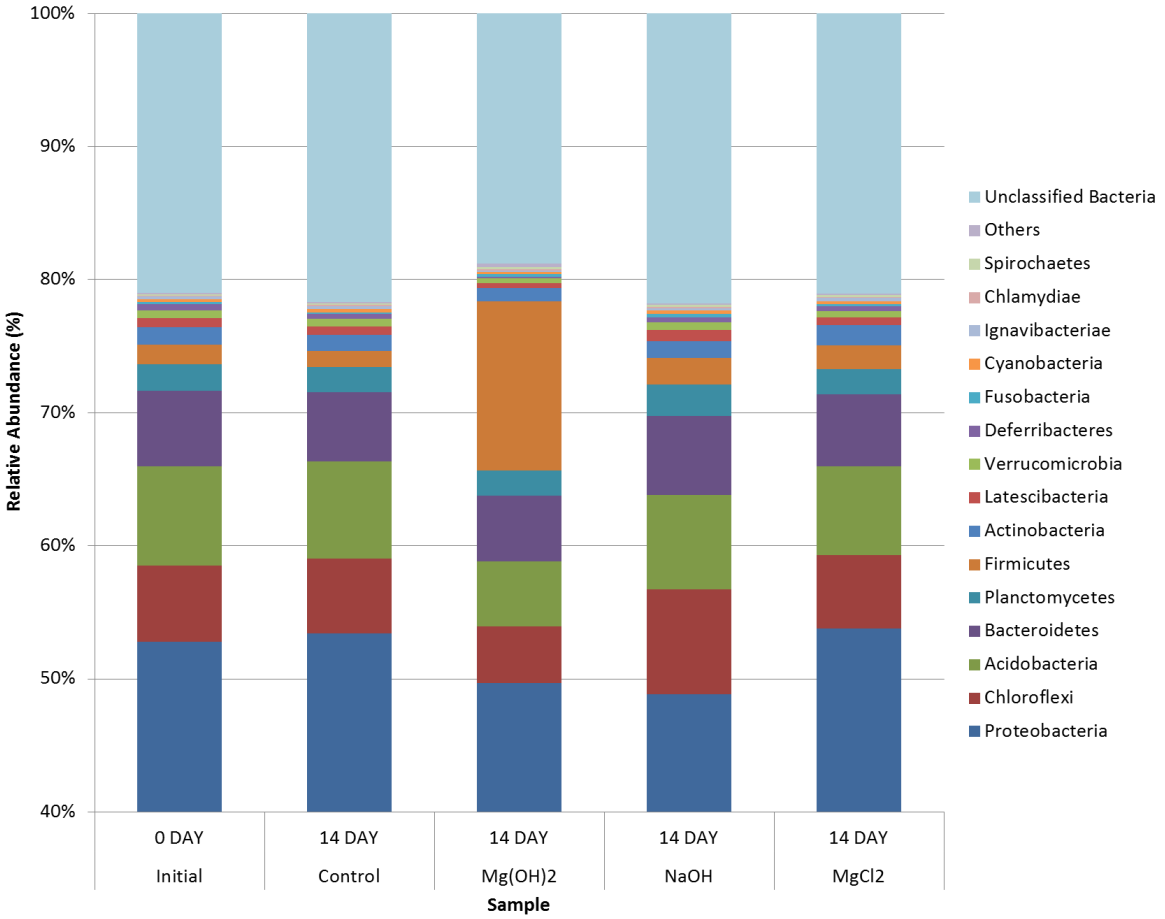

Figure 5-11 Bacterial composition in the sediment samples at the dominant bacterial phyla level. Initial: original sediment samples before incubation; 14DAY: sediment samples of each group after the 14-day incubation.

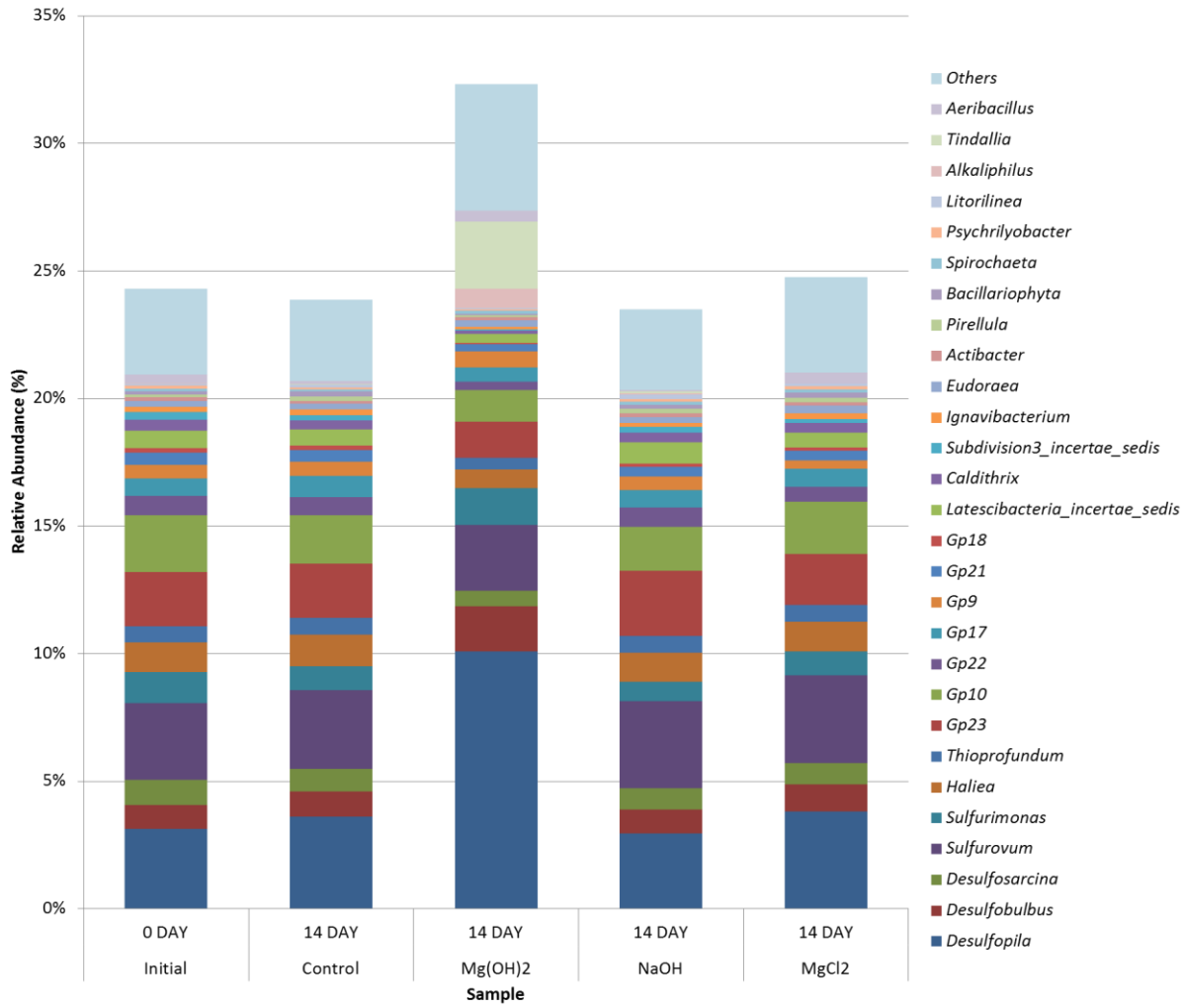

Figure 5-12 Bacterial composition in the sediment samples at the dominant bacterial genera level. Initial: original sediment samples before incubation; 14DAY: sediment samples of each group after the 14-day incubation.

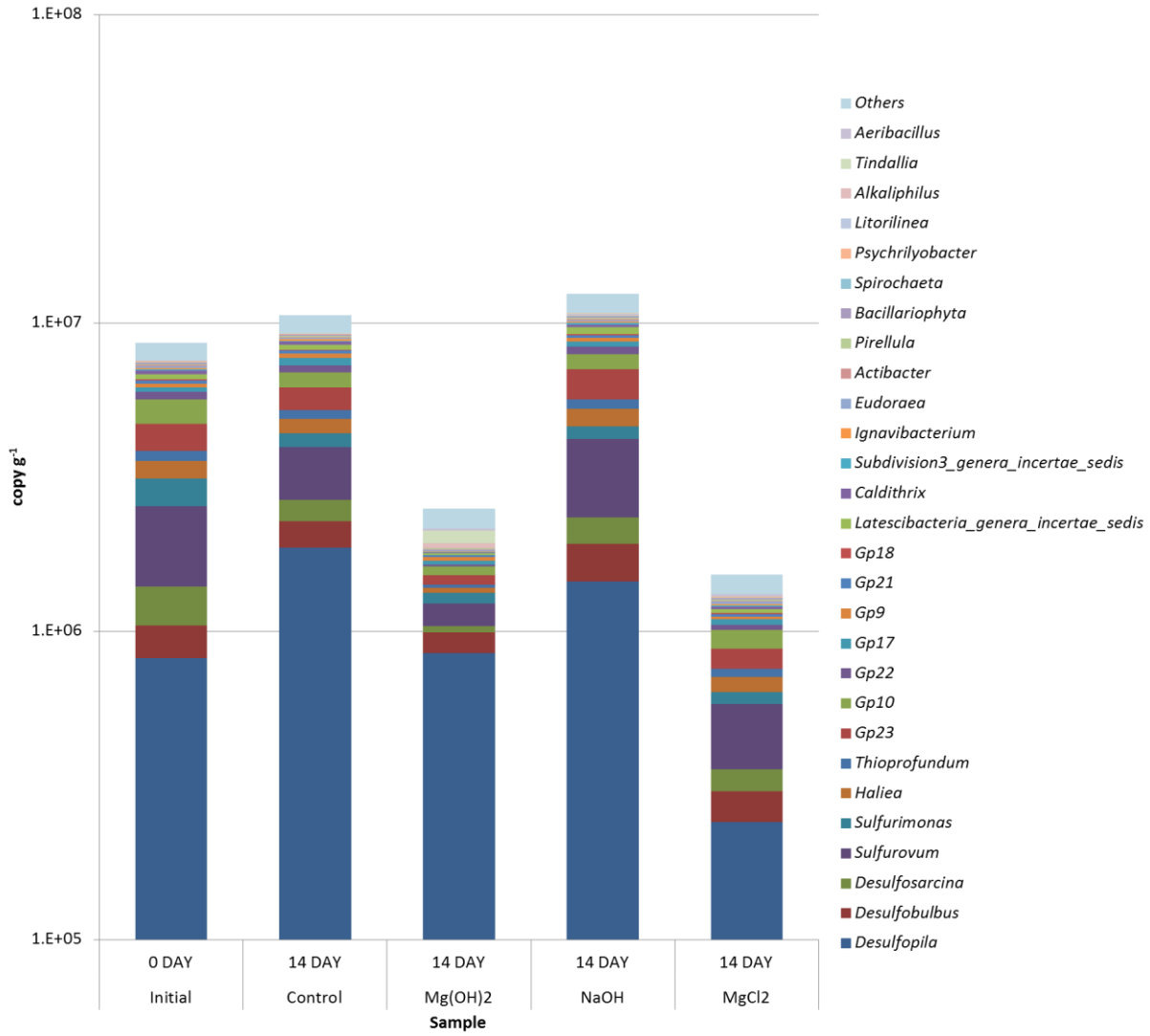

Figure 5-13 Quantification of bacterial composition in the sediments at the dominant bacterial genera level. Y axis is the copy number of bacterial 16S rRNA gene of dominant genera. Initial: original sediment samples before incubation; 14DAY: sediment samples of each group after the 14-day incubation.

5.3.5 Statistical Analysis of the Environment Factors on the Bacterial Communities

Statistical analysis was performed to elucidate the effect of Mg^{2+} ions and pH for SRB (the copy number of 16S rRNA gene of each genus *Desulfobulbus*, *Desulfopila*, *Desulfsarcina*) and the other bacteria belong to Epsilon- and Gamma-proteobacteria and Acidobacteria. A multiple regression (linear model) using two explanatory variables pH and Mg^{2+} ions was applied to

summary these effects to bacterial community (Table 5-4). The genera *Desulfsarcina*, *Desulfobulbus* and *Desulfopila* which are the typical species of SRB, only have a significant negative correlation with Mg^{2+} ions. Moreover, the genera *Sulfurovum*, *Sulfurimonas* and *Thioprofundum* that belong to Proteobacteria, Acidobacteria *Gp10* and *Gp23* that belong to Acidobacteria also showed significant negative correlation with Mg^{2+} ions rather than pH. While, a significant positive correlation was observed between the pH and the genus Tindallia belong to Firmicutes. That is, Mg^{2+} ions had a clear influence to the generation of H_2S and SRB than that of pH (Table 5-5a & 5-5b).

The environmental factors (pH, Mg^{2+}, H_2S) and the dominant genera were used to conduct the redundancy analysis (RDA) to illuminate the relationship among them. The Figure 5-14 showed the RDA result after the 14-day incubation, the first two axis (RDA1 and RDA2) by three parameters (H_2S, pH and Mg^{2+}) explained 97.01% and 2.112% variation of the bacterial communities, respectively. Both pH and Mg^{2+} showed negative correlation with RDA1 axis (r=-0.123, *P*=0.069 and r=-0.808, *P*=0.088, respectively), and positive correlation with the RDA2 axis (r=0.992, *P*=0.069 and r=0.589, *P*=0.088, respectively). While, H_2S was positively correlated with RDA1 axis (r=0.266, *P*=0.084), but was negatively correlated with RDA2 axis (r=-0.964, *P*=0.084). That is, pH and Mg^{2+} were gradually decreased, but H_2S was gradually increased from left to right on the RDA1 axis. Moreover, the arrow of pH and Mg^{2+} were longer than that of H_2S, indicated that pH and Mg^{2+} was the major factor affecting the bacteria community in the sediment

Table 5-5a Results of liner model analysis between explanatory variables (pH and Mg^{2+} concentration) and objective variables (bacteria belong to phylum Proteobacteria)

Delta-Proteobacteria

Formula: logDesulfopila~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.5812	0.2178	-2.669	0.0219*
pH	0.1537	0.2178	0.706	0.4950

Residual standard error: 0.7427 on 11 degrees of freedom

F-statistic: 5.333 on 2 and 11 DF, **p-value**: 0.02403

Formula: logDesulfobulbus~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.72542	0.20772	-3.492	0.00504**
pH	-0.08529	0.20772	-0.411	0.68925

Residual standard error: 0.7083 on 11 degrees of freedom

F-statistic: 6.616 on 2 and 11 DF, **p-value**: 0.01299

Formula: logDesulfosarcina~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.8806	0.2477	-3.554	0.00452**
pH	-0.5080	0.2477	-2.051	0.06491

Residual standard error: 0.8447 on 11 degrees of freedom

F-statistic: 6.587 on 2 and 11 DF, **p-value**: 0.01316

Epsilon-Proteobacteria

Formula: logSulfurovum~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.8435	0.2468	-3.418	0.00575**
pH	-0.4593	0.2468	-1.861	0.08965

Residual standard error: 0.8415 on 11 degrees of freedom

F-statistic: 6.012 on 2 and 11 DF, **p-value**: 0.0172

Formula: logSulfurimonas~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.67203	0.20259	-3.317	0.00686**
pH	-0.09957	0.20259	-0.491	0.63274

Residual standard error: 0.6908 on 11 degrees of freedom

F-statistic: 5.869 on 2 and 11 DF, **p-value**: 0.01843

Gamma-Proteobacteria

Formula: logHaliea~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.9205	0.2486	-3.703	0.00349**
pH	-0.5821	0.2486	-2.342	0.03906*

Residual standard error: 0.8477 on 11 degrees of freedom

F-statistic: 7.343 on 2 and 11 DF, **p-value**: 0.009427

Formula: logThioprofumdum~STDlogMg+STDpH

Coefficients

	Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-0.8703	0.2438	-3.570	0.00439**
pH	-0.5213	0.2438	-2.138	0.05577

Residual standard error: 0.8312 on 11 degrees of freedom

F-statistic: 6.711 on 2 and 11 DF, **p-value**: 0.01244

The explanatory variable Mg^{2+} concentration was converted to logarithm values, then it was standardized as the mean 0 and the variance 1. The pH was also standardized as the mean 0 and the variance 1. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

Table 5-5b Results of liner model analysis between explanatory variables (pH and Mg^{2+} concentration) and objective variables (bacteria belong to phylum Firmicutes and Acidobacteria)

Firmicutes					**Acidobacteria**				
Formula: logAlkaliphilus~STDlogMg+STDpH					**Formula: logGp10~STDlogMg+STDpH**				
Coefficients					Coefficients				
	Estimate	Std. Error	t-value	Pr(>|t|)		Estimate	Std. Error	t-value	Pr(>|t|)
logMg	77.19	105.37	0.733	0.504	logMg	-0.8261	0.2421	-3.413	0.0058**
pH	-41.57	57.52	-0.723	0.510	pH	-0.5436	0.2421	-2.246	0.0462*
Residual standard error: 0.2271 on 4 degrees of freedom					Residual standard error: 0.8254 on 11 degrees of freedom				
F-statistic: 0.5878 on 2 and 4 DF, **p-value**: 0.5973					F-statistic: 6.329 on 2 and 11 DF, **p-value**: 0.01482				
Formula: logTindallia~STDlogMg+STDpH					**Formula: logGp23~STDlogMg+STDpH**				
Coefficients					Coefficients				
	Estimate	Std. Error	t-value	Pr(>|t|)		Estimate	Std. Error	t-value	Pr(>|t|)
logMg	-1.4305	1.8980	-0.754	0.4756	logMg	-0.9643	0.2524	-3.821	0.00284**
pH	2.2082	0.7963	2.773	0.0276*	pH	-0.5428	0.2524	-2.151	0.05459
Residual standard error: 0.8129 on 7 degrees of freedom					Residual standard error: 0.8606 on 11 degrees of freedom				
F-statistic: 16.61 on 2 and 7 DF, **p-value**: 0.0022					F-statistic: 7.566 on 2 and 11 DF, **p-value**: 0.008576				

The explanatory variable Mg^{2+} concentration was converted to logarithm values, then it was standardized as the mean 0 and the variance 1. The pH was also standardized as the mean 0 and the variance 1. Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

The genera *Desulfobulbus*, *Desulfopila*, *Desulfsarcina*, *Haliea*, *Thioprofundum*, *Sulfurovum*, *Sulfurimonas*, Acidobacteria *Gp10* and Acidobacteria *Gp23* located on the positive side of RDA1 axis were negatively correlated with pH and Mg^{2+} content (Figure 5-14). On the contrary, *Alkaliphilus* and *Tindallia* located on the negative side of RDA1 axis were positively correlated with pH and Mg^{2+} content, clearly separated from the other bacteria (Figure 5-14). The sit 5,6,7,8 represented $Mg(OH)_2$ addition group, located on the negative side of RDA1 axis, and positively correlated with pH and Mg^{2+} content. Moreover, only the genera *Alkaliphilus* and *Tindallia* gathered at these positions of $Mg(OH)_2$ addition group on the RDA plot, which were corresponding to the significant increase detected in the $Mg(OH)_2$ addition group after the incubation by using the NGS method.

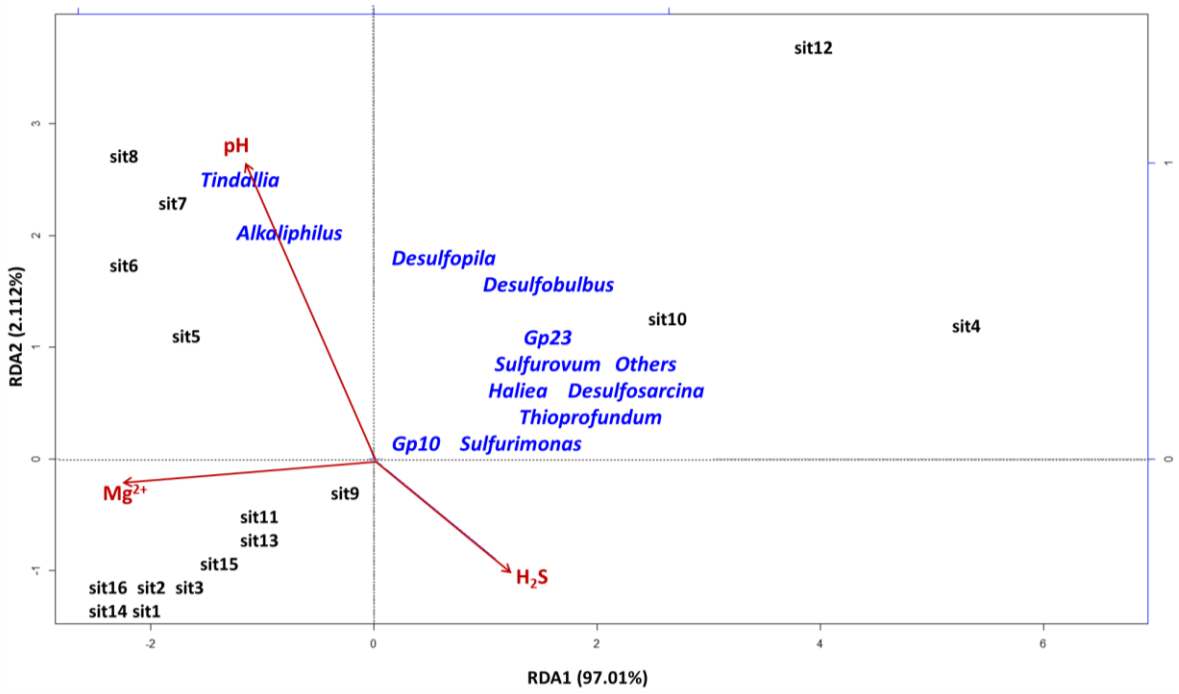

Figure 5-14 Redundancy analysis (RDA) plots of the relationship among H_2S, addition factors (pH, Mg^{2+}) and bacterial community in the sediments after the 14-day incubation. Red arrows, H_2S and addition factors (pH, Mg^{2+}); Blue letters, dominant genera; sit 1, 2, 3, 4: control group; sit 5, 6, 7, 8: $Mg(OH)_2$ addition group; sit 9, 10, 11, 12: NaOH addition group; sit 13, 14, 15, 16: $MgCl_2$ addition group.

5.4 DISCUSSIONS

In this study, the significant decrease of the copy number of *dsrA* gene and that of the H_2S content after the 14-day incubation were also observed in control group. The most possible explanation might be attributed to the used sediments, which were collected in November of 2015. Taguchi *et al.* (2014) conducted a series of numerical simulations about the rise and fall of the hypoxic water mass in Omura Bay using the observation data at several monitoring points. They found that the hypoxia in the bottom layer of Omura Bay would continue from June until September. The highest stage of the benthic hypoxia appeared in mid-August and the recovery process of oxygen by the inflowing oceanic water with rich oxygen turned out in the early October, the hypoxia in the bottom layer was essentially eliminated until November. Furthermore, the recovery of oxygen declined the sulfate reduction activity in the bottom layer, and demonstrated that the organic decomposition process by aerobic bacteria was also carrying out simultaneously in the sediments. Through the sulfate reduction process in the sediment in summer season, the dissolved organic matter (DOC) could be actively consumed which as the food source of the sulfate-reducing bacteria (SRB). Due to the condition of the sediment surface

in Omura bay had already changed from anoxic to aerobic, many aerobic bacteria actively consumed DOC though the SRB activity was decreased. Therefore, it was thought that the food source in the collected sediment samples became shortage for the growth of SRB during the incubation of the experiment. Namely, the increase of the mortality rate of SRB due to the food resource shortage might be the main factor that results in the decrease of the *dsrA* gene and H_2S in the sediments of control group.

However, the copy number of three SRB genera *Desulfobulbus*, *Desulfopila* and *Desulfsarcina* calculated by the copy number of total bacterial 16S rRNA gene were increased in the control group after the 14-day incubation. This result is distinct to the copy number of *dsrA* gene detected by specific *dsrA* gene primer set Grp3. Cook *et al.* (2008) had succeed to design TaqMan probes with primers to specifically target the *dsrA* gene of Group 1A SRB, Group 1B SRB, Group 2A SRB and Group 3A SRB. Moreover, enumeration for these organisms using quantitative real-time PCR method was also achieved. They used Grp1 and Grp1B primer sets to target Desulfobulbus-like species, Grp2 primer sets were used to target Desulfovibrio-like species and Grp3 primer sets to target *Desulfovibrio* species. Spence *et al.* (2008) also succeeded to develop the real-time PCR of SYBR assay for Group 1, Group 2 and Group 3 of SRB from manure samples. In this chapter, the primer set Grp3 was applied to specifically amplify a 119 bp target fragment of *dsrA* gene of SRB. On the other hand, the real-time PCR with the primer sets for Grp1 and Grp2 never showed the normal amplification curves of the samples although the several PCR conditions were examined. Therefore, a portion of SRB could be only detected and measured in present study. Considering the consistency with the experimental results on the decrement in H_2S and the copy number of *dsrA* gene, it is reasonable that the activity of the other major groups of SRB might be inhibited by the addition of $Mg(OH)_2$ as well as Grp3 SRB, although the strength of the effect for each group of SRB might be different.

The results of multivariate linear regression analysis shown in Table 5-4 clearly elucidated that Mg^{2+} ion is the major factor rather than pH on affecting the population of SRB (*Desulfobulbus*, *Desulfopila* and *Desulfsarcina*) and the generation of H_2S. Xiong *et al.* (2012) suggested that some ions correlated with pH, such as Mg^{2+}, showed significantly positive correlation with the relative abundance of Firmicutes. In this study, the high abundance of the genera *Alkaliphilus* and *Tindallia* were observed only in $Mg(OH)_2$ and NaOH addition groups at the end of the incubation (Table 5-3). Moreover, these two genera were almost no detected in $MgCl_2$ addition group during the incubation. Compared with Mg^{2+} ions, the pH might be more important influencing factor to the growth of Firmicutes, which the significantly positive correlation between the genera of Firmicutes and pH was observed after the incubation (Figure

5-13). These results mentioned above indicated that the influence of Mg^{2+} ions or pH on the growth of bacteria and the composition of bacterial community in the sediments might be dependent on the characteristic of the bacteria and their habitat environment.

Mg^{2+} is the most abundant divalent cation in the living cells and plays a key role in cellular metabolism. Since Mg^{2+} ions need bind with specific Mg^{2+} membrane transporters to transport Mg^{2+} into the cells, three distinct classes of Mg^{2+} transporters CorA, MgtE and MgtA has been identified in gram-negative bacteria *Salmonella enterica* serovar Typhimurium (Hmiel *et al.* 1986, 1989 & Smith *et al.* 1995). Snavely *et al.* (1991) proposed that CorA transports Mg^{2+} under normal Mg^{2+} levels, whereas MgtA and MgtB transport Mg^{2+} when bacteria faces low Mg^{2+} condition. Subramani *et al.* (2016) also confirmed that the activity of MgtA will be stimulated when the free Mg^{2+} concentration below to 10 μM and it is strongly inhibited when the free Mg^{2+} concentration above 1 mM.

The concentration of free Mg^{2+} ions in cytoplasm within *Escherichia coli* was found over a range of 0.1 Mm to 1 mM (Silver & Clark 1971; Froschauer *et al.* 2004). Furthermore, Webb (1966) confirmed that *E. coli*, which is a typical Gram-negative bacteria, had maximum growth rate at the concentration of Mg^{2+} ions in the medium from 0.00125 mM to 0.625 mM. Moreover, *E. coli* could uptake and utilize Mg^{2+} ions in the culture medium completely when the concentration of this cation maintained at this low range. In this study, the Mg^{2+} concentration in the sediments of $Mg(OH)_2$ and $MgCl_2$ addition group was significantly higher than that of in the bacterial cells, which might inhibit the activity of Mg^{2+} transporters and led to a decrease of Mg^{2+} concentration in intracellular. Moreover, the discharge mechanism of reverse transport of Mg^{2+} concentration even might be activated due to the excess state of Mg^{2+} in the microbial cells. The increased mortality of the bacterial cells might affect the effectiveness of sulfate reduction in the sediments. On the other hand, the dissimilatory sulfate reduction mediated by SRB requires the consumption of a single ATP molecule to convert sulfate to APS. But the activity of ATP is closely correlated with Mg^{2+} ion, since Mg^{2+} ion plays an essential role in the biological activity of ATP, which must be bound to Mg^{2+} ions to be biologically active. So less available of ATP resulted in the inhibition of sulfate reduction, and then the generation of H_2S was also reduced. Further study is necessary to detailed investigate the effect of Mg^{2+} ion on bacteria.

5.5 CONCLUSIONS

(1) After the 14-day incubation, the pH value of the sediments in $Mg(OH)_2$ addition group were increased and exceeded 8.5, the H_2S content in interstitial water and the abundance of SRB were simultaneously decreased in $Mg(OH)_2$ addition group. A similar decrease of the H_2S

content and that of SRB were observed in NaOH and $MgCl_2$ addition groups, although the pH of the sediments in these two groups were maintained around 7.94 ±0.05 and 7.35 ±0.05 respectively after the 14-day incubation. The multivariate linear regression analysis clearly presented that the both Mg^{2+} ions and the alkalization reduced the generation of H_2S by the SRB activity.

(2) The NGS analysis revealed the changes in composition of bacterial community. The genera belong to Delta-proteobacteria was decreased in $Mg(OH)_2$ and $MgCl_2$ addition group, but increases of *genera Alkaliphilus, Tindallia* were observed only in $Mg(OH)_2$ addition group.

(3) The RDA analysis revealed that after the 14-day incubation, SRB within Proteobacteria negatively correlated with pH and Mg^{2+}, but positively correlated with H_2S. While, only Firmicutes showed positive correlation (P<0.01) with pH.

(4) The results of this study indicated that adding $Mg(OH)_2$ to the sediment can inhibit the activity of SRB and the generation of H_2S, meanwhile also can affect the composition of bacterial communities in the sediments. Moreover, both alkalization and Mg^{2+} ion were the major factors, which interacted each other to achieve the inhibition effect of $Mg(OH)_2$.

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6 SUMMARIES

The remediation effectiveness of $Mg(OH)_2$ on sediment environment and the influences on the bacterial communities in the sediments, the effect of magnesium ion (Mg^{2+}) and alkalization on the generation of H_2S and the microbial community in the sediments are discussed in this doctoral thesis. The main conclusions are summarized in this chapter.

In chapter 3, the addition of $Mg(OH)_2$ raised the pH of the sediment over to 8 which weakened the sulfate-reducing activity of SRB. Hence, the generation of AVS and that of H_2S in sediments were inhibited. Moreover, the increase of the DOC observed in treatment group sediment also strongly indicated the decrease of the activity of SRB, because DOC is a major substrate of SRB. In summary, the usefulness of $Mg(OH)_2$ for the sediment remediation was ensured in this study.

In chapter 4, after the laboratory incubation, the pH value of the sediments and DOC content of interstitial water in treatment group have increased, while the AVS content have decreased. The NGS analysis revealed the changes in composition of bacterial community. Addition of $Mg(OH)_2$ in sediments showed a decrease number of Delta-proteobacteria and increase number of Firmicutes. The RDA analysis also revealed that SRB within Proteobacteria showed negative correlation (P<0.001) with pH and positive correlation (P<0.001) with AVS. However, Firmicutes positively correlated (P<0.001) with pH, but negatively correlated (P<0.001) with AVS. Moreover, the genera *Fusibacter*, *Alkaliphilus* and *Tindallia* within Firmicutes also showed positive correlation with DOC (P<0.001). The results of this chapter indicated that adding $Mg(OH)_2$ to the sediment can inhibit the activity of SRB and reduce the generation of sulfide, at the same time it affects the composition of bacterial communities by accelerating the growth of alkaliphilic bacteria.

In chapter 5, after the laboratory incubation, the pH value of the sediments in $Mg(OH)_2$ and NaOH addition group were increased and exceeded 8.0, but the H_2S content in interstitial water and the abundance of SRB were simultaneously decreased. The NGS analysis revealed the changes in composition of bacterial community. The genera belong to Delta-proteobacteria was decreased in $Mg(OH)_2$ and $MgCl_2$ addition group, but increases of genera *Alkaliphilus*, *Tindallia* were observed only in $Mg(OH)_2$ addition group. The RDA analysis revealed that after the 14-day incubation, SRB within Proteobacteria negatively correlated with pH and Mg^{2+}, but positively correlated with H_2S. While, only Firmicutes showed positive correlation (P<0.01)

with pH. The results of this study indicated that adding $Mg(OH)_2$ to the sediment can inhibit the activity of SRB and the generation of H_2S, meanwhile also can affect the composition of bacterial communities in the sediments. Moreover, both of alkalization and Mg^{2+} ion might be the major factors for the inhibition effect of $Mg(OH)_2$.

For the future work, the hypothesis about the purification mechanism of $Mg(OH)_2$ to the sediments is discussed. Due to the season cycle, the water column in the field sites will show the different characteristics, such as the water temperature, dissolved oxygen (DO), salinity and so on. Due to these differences, the $Mg(OH)_2$ sprayed on the sediments will play different roles in the process of sediment purification. To certificate the purification effects of $Mg(OH)_2$ in the sediments, the field experiments for spraying the $Mg(OH)_2$ to the sediments of fish ponds area last for one year, and measure the chemical and biological parameters in the water column and sediments to analyze the different functions of the $Mg(OH)_2$ during the seasonal cycle.

APPENDIX

On-site Verification Test of Sediment Improvement Technology with Magnesium Oxide and Magnesium Hydroxide-Environmental Technology Verification (ETV) Program from Ministry of the Environment

***** Data of ETV program to make all tables and all figures were provided from Ube Material Industries Ltd.

Introduction

The on-site verification test of magnesium oxide (MgO) and magnesium hydroxide ($Mg(OH)_2$) were conducted in Omura Bay, Japan in Environmental Technology Verification (ETV) Program by Ministry of the Environment.

This verification technology expects to suppress the growth of sulfate-reducing bacteria (SRB) and the generation of hydrogen sulfide (H_2S) by keeping the surface layer of sediment weak alkaline at pH 8.0 or more by spraying MgO and $Mg(OH)_2$. It is known that the activity and growth rate of SRB could be suppressed when the pH of the culture medium maintained at alkaline range (O'Flaherty *et al.* 1998). Therefore, it is expected to raise the pH of sediments above 8.0 by spraying MgO and $Mg(OH)_2$ to suppress the activity of SRB and the generation of H_2S. In addition, H_2S has strong toxicity and can induce fatal death of benthic organisms. Since these benthic organisms living in the surface layer of sediments play an important role in material circulation between seawater and sediment, their death will result in a declines of material circulation in the marine area. Therefore, it is expected to maintain an habitable environment for benthic organisms and form a natural material circulation by suppressing the activity of SRB and the generation of H_2S by spraying MgO and $Mg(OH)_2$.

Compared to other purification methods such as dredging (Manap & Voulvoulis 2015), tillage and sand capping, spraying MgO and $Mg(OH)_2$ method applied in this verification program is no large-scale facilities, no secondary pollution problems and no processing for dredged sludge, the cost is also relatively inexpensive. Moreover, compared to other alkaline materials such as lime, MgO and $Mg(OH)_2$ are extracted from marine and the pH of them rise slowly that are highly safe to benthic organisms and less risk to sedimentary environment (Nishno & Kawauchi 2003).

Consequently, in this verification test, keeping the pH of surface layer of sediments (about 2

cm) at 8.0 or higher by spraying MgO and $Mg(OH)_2$, the effect of suppressing the growth of SRB and the generation of H_2S are expected. Moreover, keeping the pH of the sediment in test group sprayed MgO and $Mg(OH)_2$ at 8.0 or higher, and leading the content of H_2S lower than control group (no sprayed) are used as the standard of the objective.

In this chapter, the data of this verification test were used to statistically analyze the effect of MgO and $Mg(OH)_2$ on the suppression of H_2S generation, the suppression of total sulfides production and the influences on benthic organisms.

Materials and Methods

Study Site

This verification test was carried out at the place near Urago (32°49′57″N 129°50′52″E) of Togitsu-machi in Nagasaki Prefecture located in the inner part of Oumra Bay (Figure A-1). The verification test is scheduled to be carried out on the bottom of the sea at the water depth of about 5 m near the Togitsu port of Omura Bay, and the test area is assumed to be about 200 m^2 (10 m × 10 m × 2 sections). The sediment of Omura Bay is composed of very fine particles and the median particle size of sediment at the central part of the bay is 4 μm.

Experiment setup

Test area sprayed with MgO and $Mg(OH)_2$ and control area (no sprayed) were set up to confirm the effect of MgO and $Mg(OH)_2$. The size of the test area was 3 × 3 m and the distance of the test area was 3 m. For the test areas, different spraying times were set to confirm the effective spraying method. That is, the area “MgO test 1” with one time spraying, “MgO test 2” and “$Mg(OH)_2$ test” with two times spraying were set (Figure A-2).

The MgO material (Ube Materials Co., Yamaguchi, Japan) applied in this verification program has maximum particle diameter of 0.15mm and density of 3.58 g cm^3. The $Mg(OH)_2$ material (clear water, Ube Materials Co., Yamaguchi, Japan) has granules of 2mm diameter.

Sediment, Benthic Organisms and Water Quality Surveys

For the sediment survey, the pH of sediments, total sulfide (AVS), Ignition Loss (IL), Oxidation-Reduction Potential (ORP), Chl-a and the H_2S in pore water were analyzed.

For the benthic organisms survey, the species of benthos were identified, the number of individuals and wet weight were measured. For the water quality survey, continuous observation and vertical observation were carried out simultaneously from June to October, and the water

temperature, salinity and dissolved oxygen concentration were measured.

The equipment used in these surveys, the measurement methods and the schedules of observation survey were deeply described in “Verification Report on Water Environment Improvement Technologies for Enclosed Coastal Seas Field of 2015 ETV Program”.

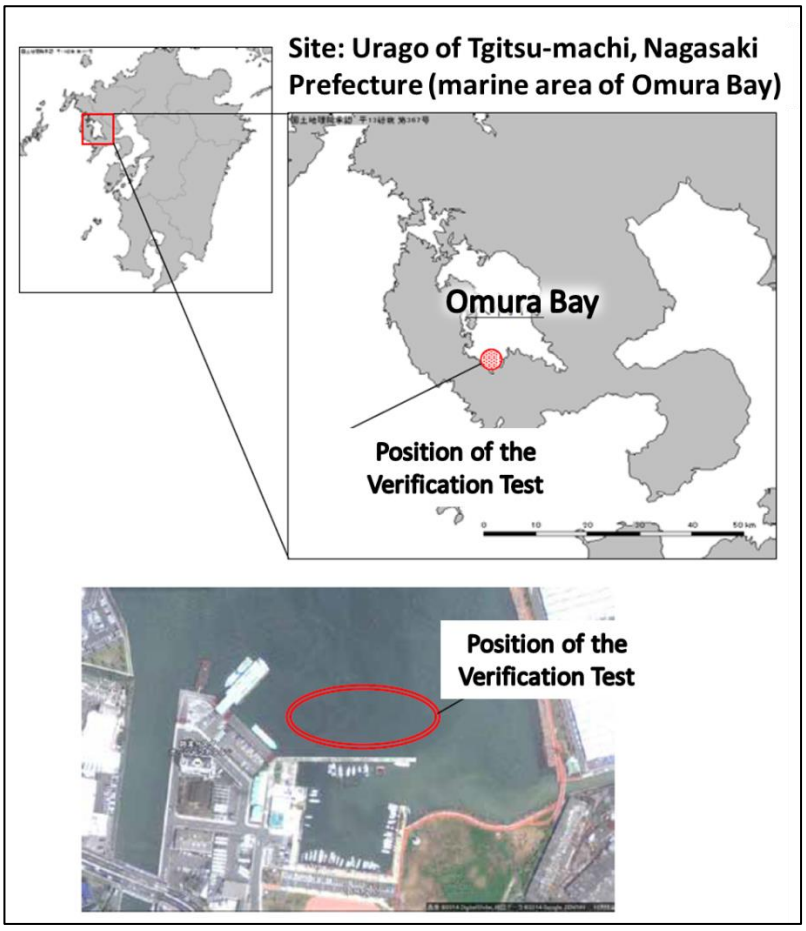
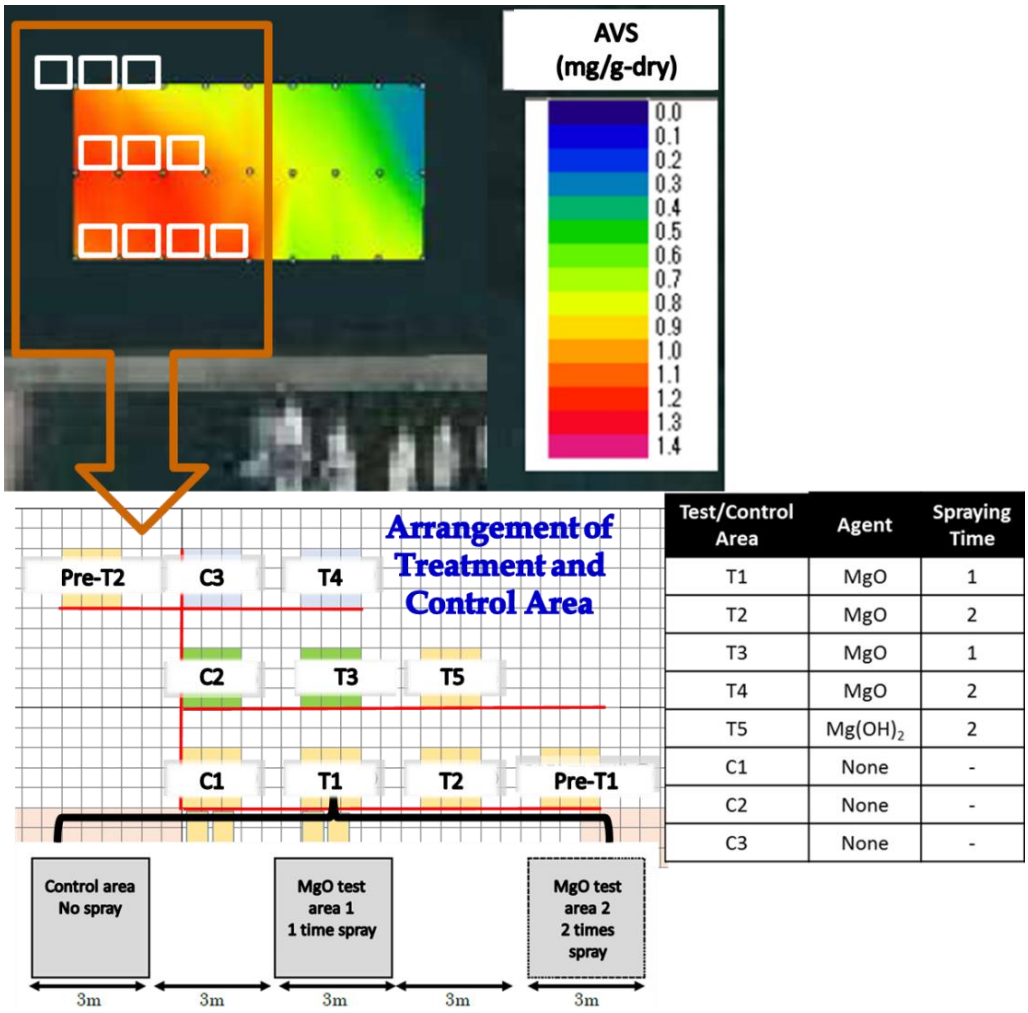


Figure A-1 Site of the Verification Test (Omura Bay, Nagasaki Prefecture)
(From Verification Report on Water Environment Improvement Technologies for Enclosed Coastal Seas Field of 2015 ETV Program)



Test/Control Area	Agent	Spraying Time
T1	MgO	1
T2	MgO	2
T3	MgO	1
T4	MgO	2
T5	$Mg(OH)_2$	2
C1	None	-
C2	None	-
C3	None	-

Figure A-2 Image of Placement of Test Area and Control Area

(From Verification Report on Water Environment Improvement Technologies for Enclosed Coastal Seas Field of 2015 ETV Program)

Statistical Analysis

The relationship between the sediment conditions (pH of sediments, content of H_2S and AVS) and the impact factors of test (seasonal changes and the spraying of MgO and $Mg(OH)_2$) were analyzed by a linear model analysis. Moreover, the relationship between the impact factors of test and the benthic organisms (species and individuals), the relationship between the sediment conditions and the benthic organisms were also analyzed by linear model analysis.

Results

The changes of pH and AVS of sediments, H_2S of pore water and the benthic organisms

The results pH, AVS, H_2S and the benthic organisms were described in "Verification Report on Water Environment Improvement Technologies for Enclosed Coastal Seas Field of 2015 ETV

Program”.

Table A1 Temporal changes of pH in sediments

Group		22-Jun	7-Jul	21-Jul	7-Aug	19-Aug	3-Sep	16-Sep	20-Oct	4-Dec
		7.70	9.49	8.21	8.08	7.80	7.71	7.78	7.70	7.69
	MgO Addition	7.58	9.44	8.33	8.03	8.00	7.74	7.75	7.67	7.63
Treatment 1		7.54	9.53	8.41	8.10	7.75	7.76	7.64	7.66	7.70
	Average	7.61	9.49	8.32	8.07	7.85	7.74	7.72	7.68	7.67
	STDEV	0.08	0.05	0.10	0.04	0.13	0.03	0.07	0.02	0.04
		7.54	9.12	8.28	7.98	8.56	8.28	8.14	8.63	7.81
	MgO Addition	7.65	9.16	8.21	7.95	8.64	8.16	8.29	8.52	7.92
Treatment 2		7.61	9.20	8.14	7.89	8.58	8.16	8.03	8.58	7.88
	Average	7.60	9.16	8.21	7.94	8.59	8.20	8.15	8.58	7.87
	STDEV	0.06	0.04	0.07	0.05	0.04	0.07	0.13	0.06	0.06
		7.63	8.29	8.28	7.93	8.22	7.81	8.20	7.72	7.60
	Mg(OH)2 Addition	7.68	8.43	7.89	8.00	8.04	7.92	8.05	7.70	7.67
Treatment 3		7.68	8.69	7.92	7.89	8.14	7.91	8.28	7.80	7.68
	Average	7.66	8.47	8.03	7.94	8.13	7.88	8.18	7.74	7.65
	STDEV	0.03	0.20	0.22	0.06	0.09	0.06	0.12	0.05	0.04
		7.57	7.65	7.64	7.48	7.52	7.61	7.35	7.38	7.34
	Non Addition	7.58	7.67	7.74	7.50	7.50	7.58	7.40	7.37	7.29
Control		7.58	7.68	7.66	7.52	7.43	7.55	7.38	7.38	7.30
	Average	7.58	7.67	7.68	7.50	7.48	7.58	7.38	7.38	7.31
	STDEV	0.01	0.02	0.05	0.02	0.05	0.03	0.03	0.01	0.03

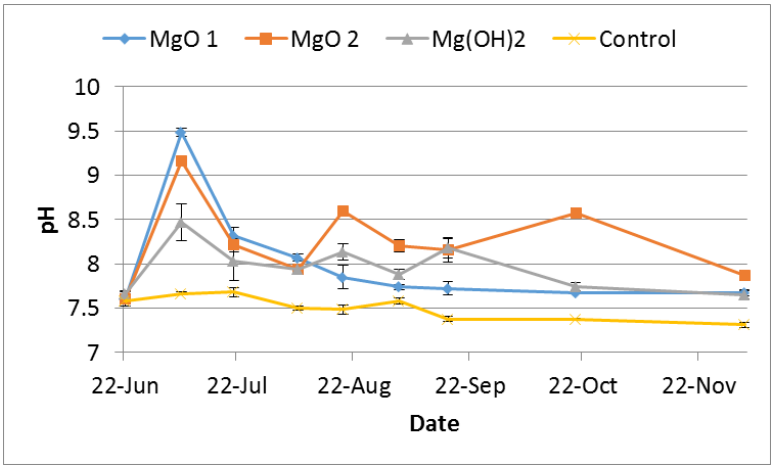


Figure A1 Temporal changes of pH in sediments

Table A2 Temporal changes of ORP in sediments

Group		22-Jun	7-Jul	21-Jul	7-Aug	19-Aug	3-Sep	16-Sep	20-Oct	4-Dec
		298	150	138	207	146	201	127	175	99
	MgO Addition	227	142	159	250	147	210	150	190	124
Treatment 1		238	113	160	203	160	210	140	183	160
	Average	254	135	152	220	151	207	139	183	128
	STDEV	38	19	12	26	8	5	12	8	31
		208	178	147	262	167	188	117	171	112
	MgO Addition	295	141	179	243	153	195	136	181	158
Treatment 2		259	145	134	237	161	183	141	168	95
	Average	254	155	153	247	160	189	131	173	122
	STDEV	44	20	23	13	7	6	13	7	33
		237	179	177	240	157	164	140	192	147
	Mg(OH)2 Addition	302	178	152	216	146	174	155	157	80
Treatment 3		231	153	159	225	165	156	166	168	102
	Average	257	170	163	227	156	165	154	172	110
	STDEV	39	15	13	12	10	9	13	18	34
		190	126	140	205	156	190	97	137	112
	Non Addition	172	112	86	206	133	153	112	145	144
Control		166	104	88	173	150	161	106	122	155
	Average	176	114	105	195	146	168	105	135	137
	STDEV	12	11	31	19	12	19	8	12	22

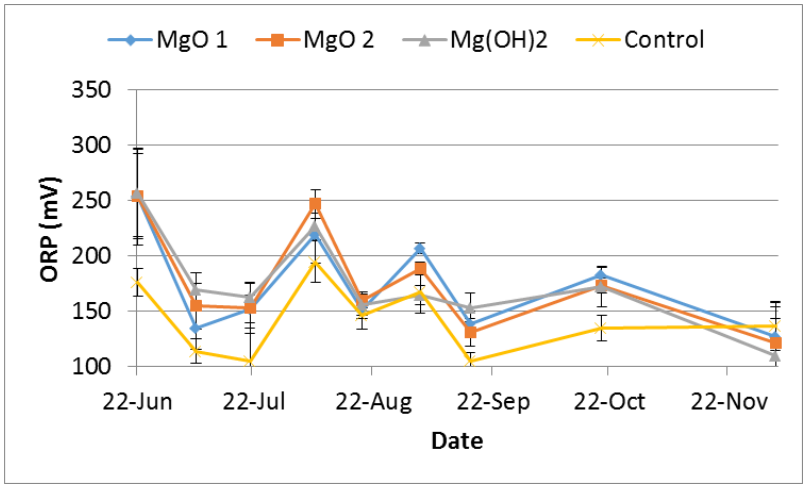


Figure A2 Temporal changes of ORP in sediments

Table A3 Temporal changes of H_2S in interstitial water

Group	H2S	22-Jun	7-Jul	21-Jul	7-Aug	19-Aug	3-Sep	16-Sep	20-Oct	4-Dec
		0.037	0.022	0.021	0.036	0.023	0.040	0.026	0.018	0.026
	MgO Addition	0.044	0.020	0.022	0.023	0.012	0.026	0.013	0.012	0.026
Treatment 1		0.049	0.022	0.020	0.027	0.017	0.028	0.029	0.010	0.020
	Average	0.043	0.021	0.021	0.029	0.017	0.031	0.023	0.014	0.024
	STDEV	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.00
		0.044	0.018	0.022	0.019	0.021	0.028	0.024	0.023	0.033
	MgO Addition	0.021	0.014	0.016	0.032	0.032	0.026	0.033	0.017	0.016
Treatment 2		0.040	0.015	0.015	0.016	0.039	0.024	0.027	0.015	0.016
	Average	0.035	0.016	0.017	0.022	0.031	0.026	0.028	0.019	0.022
	STDEV	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.01
		0.031	0.026	0.037	0.028	0.040	0.018	0.013	0.007	0.013
	Mg(OH)2 Addition	0.041	0.016	0.032	0.026	0.049	0.019	0.018	0.018	0.023
Treatment 3		0.022	0.028	0.026	0.021	0.011	0.019	0.019	0.015	0.013
	Average	0.031	0.023	0.032	0.025	0.033	0.019	0.017	0.013	0.016
	STDEV	0.01	0.01	0.01	0.00	0.02	0.00	0.00	0.01	0.01
		0.044	0.044	0.029	0.033	0.034	0.020	0.033	0.011	0.012
	Non Addition	0.048	0.054	0.040	0.028	0.023	0.035	0.038	0.017	0.015
Control		0.023	0.038	0.051	0.026	0.049	0.034	0.039	0.021	0.026
	Average	0.038	0.046	0.040	0.029	0.036	0.030	0.037	0.016	0.018
	STDEV	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.01

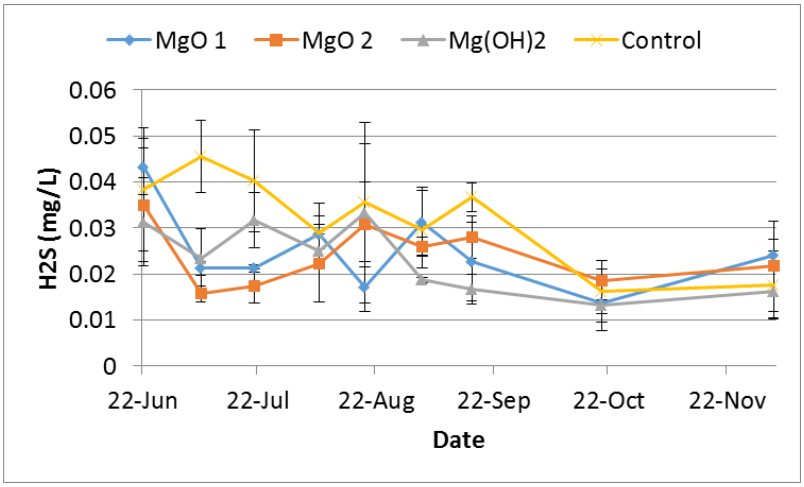


Figure A3 Temporal changes of H_2S in interstitial water

Table A4 Temporal changes of AVS in sediments

Group		22-Jun	7-Jul	21-Jul	7-Aug	19-Aug	3-Sep	16-Sep	20-Oct	4-Dec
		0.905	1.180	1.030	0.988	1.000	0.931	0.637	0.744	0.814
	MgO Addition	0.883	1.140	1.110	1.080	0.989	1.060	0.732	0.841	0.826
Treatment 1		0.934	0.988	1.350	1.090	0.931	0.612	0.609	0.727	0.554
	Average	0.907	1.103	1.163	1.053	0.973	0.868	0.659	0.771	0.731
	STDEV	0.03	0.10	0.17	0.06	0.04	0.23	0.06	0.06	0.15
		0.967	1.000	0.681	1.010	0.728	0.957	0.468	0.253	0.381
	MgO Addition	0.798	0.862	0.854	1.010	1.030	0.872	0.419	0.609	0.391
Treatment 2		1.020	0.993	0.858	0.952	0.751	0.668	0.683	0.335	0.331
	Average	0.928	0.952	0.798	0.991	0.836	0.832	0.523	0.399	0.368
	STDEV	0.12	0.08	0.10	0.03	0.17	0.15	0.14	0.19	0.03
		0.624	0.866	0.943	1.220	0.729	0.615	0.184	0.766	0.533
	Mg(OH)2 Addition	0.928	0.914	1.070	1.200	0.706	0.731	0.275	0.626	0.673
Treatment 3		0.905	1.300	1.080	1.230	0.642	0.544	0.467	0.943	0.523
	Average	0.819	1.027	1.031	1.217	0.692	0.630	0.309	0.778	0.576
	STDEV	0.17	0.24	0.08	0.02	0.05	0.09	0.14	0.16	0.08
		0.586	1.360	1.690	1.590	0.928	0.935	0.787	1.680	0.677
	Non Addition	1.590	1.320	1.590	1.820	0.974	1.060	0.364	1.440	1.060
Control		1.190	1.380	1.340	1.830	0.834	0.939	1.010	1.210	1.000
	Average	1.122	1.353	1.540	1.747	0.912	0.978	0.720	1.443	0.912
	STDEV	0.51	0.03	0.18	0.14	0.07	0.07	0.33	0.24	0.21

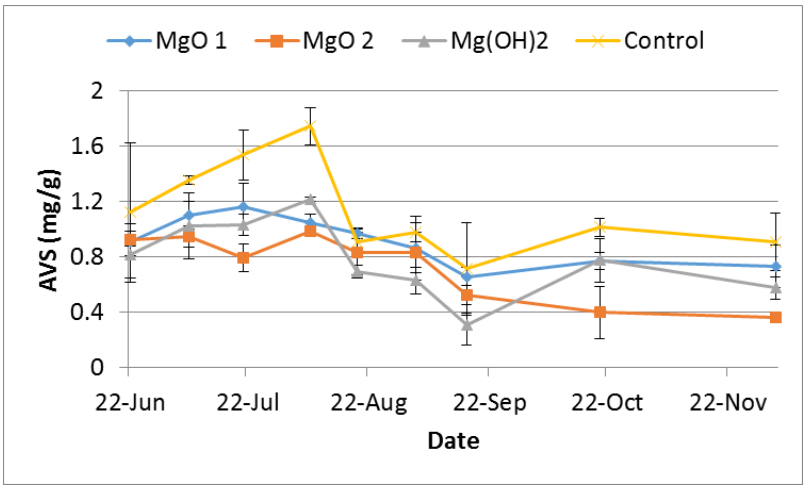


Figure A4 Temporal changes of AVS in sediments

Figure A5 Temporal changes of benthic organisms in sediments

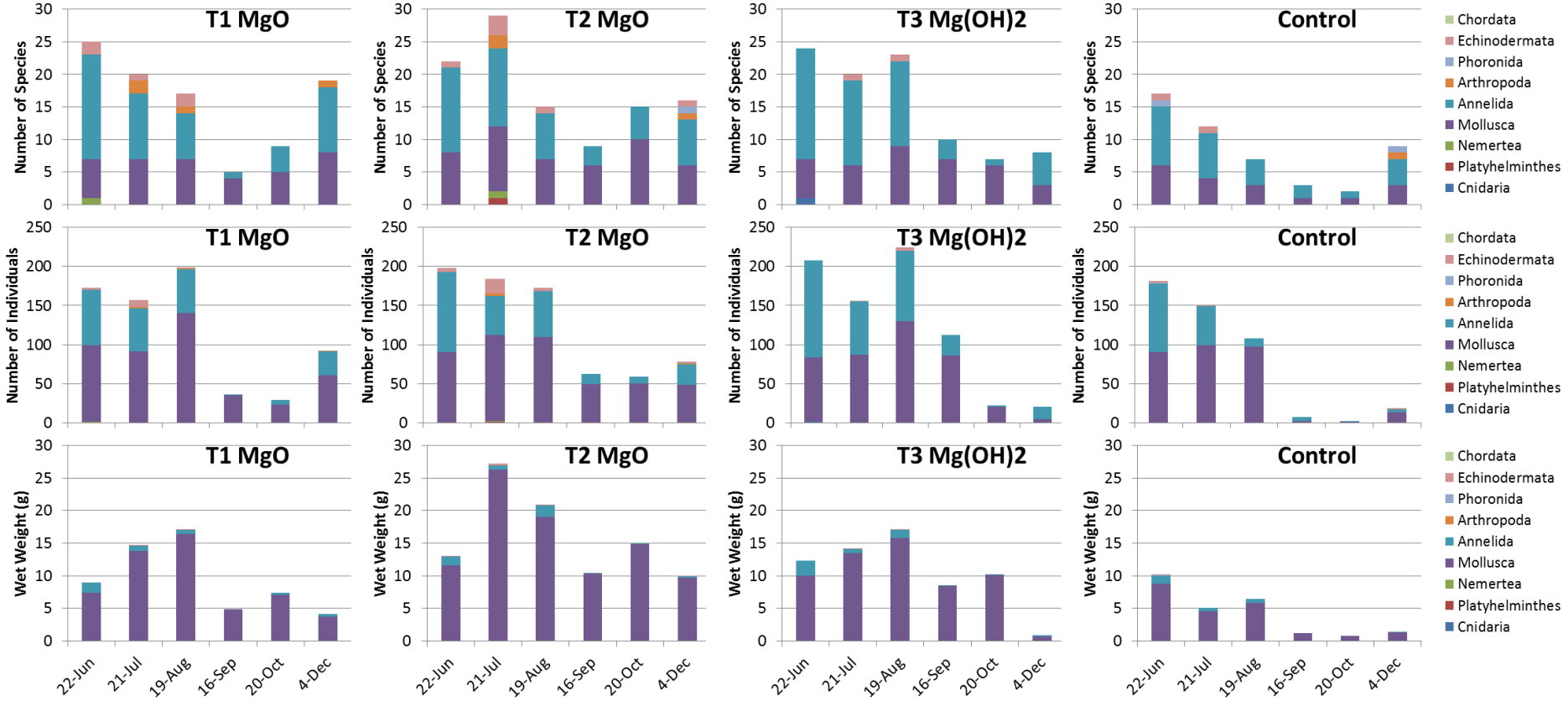


Table A5 The individual number of dominant species in this verification test.

Phylum	Class	Species	22-Jun				21-Jul				19-Aug				16-Sep				20-Oct				4-Dec			
			T1	T2	T3	C1	T1	T2	T3	C1	T1	T2	T3	C1	T1	T2	T3	C1	T1	T2	T3	C1	T1	T2	T3	C1
Mollusca	Bivalvia	*Pillucina pisidium*	15	8	18	4	5	27	15		7	8	13		1	3	6		3	3	1		11	4		
Mollusca	Bivalvia	*Macoma incongrua*	15	22	16	15	24	20	18	8	21	15	23	3	7	10	6	2	9	16	9	1	3	10	1	1
Mollusca	Bivalvia	*Theora fragilis*	55	50	44	59	50	55	46	89	106	77	81	93	21	32	68		7	20	5		37	25	3	10
Mollusca	Bivalvia	*Paphia undulata*			1	1		1				1	1		6	2	1						1			
Mollusca	Bivalvia	*Moerella rutila*		2					3		1	4	3			1	1									
Mollusca	Bivalvia	*Nitidotellina minuta*	3	3			3	2	3		2	2	2							1						
Mollusca	Bivalvia	*Moerella culter*																		2	1		2	6	1	
Mollusca	Bivalvia	Tellinidae gen sp.					4	1									1		2	2	2		2	1		
Mollusca	Bivalvia	*Paphia sp.*															3		2	3	2					
Mollusca	Gastropoda	*Reticunassa festiva*	6	1	2	10	2		2	1						1										
Annelida	Polychaeta	*Sigambra sp.*			1				2	1	2	5	1				19	2	2	2			5	15	6	1
Annelida	Polychaeta	*Notomastus sp.*	16	27	23	15	26	21	17	8	31	11	16	6		10	6		2		2	1	8	1	3	
Annelida	Polychaeta	*Lagis bocki*	1		2		4	1	4	3		1	3				1						8	5	3	
Annelida	Polychaeta	*Glycera nicobarica*	2	1	6	2		1				3	1	1					1							
Annelida	Polychaeta	*Scoletoma longifolia*	4	7	4	4	2	4	1	3		5	7													
Annelida	Polychaeta	*Scoletoma sp.*	25	51	67	52	13	16	25	33	19	31	48	3										1		
Annelida	Polychaeta	*Leitoscoloplos pugettensis*	3	5	3	3			1																	
Annelida	Polychaeta	*Phylo sp.*	3	2	1		1	1				2														
Annelida	Polychaeta	Maldanidae gen sp.	3	1	3	1	4	1	10	1			3			2										
Annelida	Polychaeta	*Chone sp.*	3	1	5	7	1		1		1		4													
Annelida	Polychaeta	*Paraprionospio cordifolia*						1												2			1	3	2	1
Echinodermata	Ophiuroidea	Amphiuridae gen sp.	2	5		2	9	16	1	1	2	5	4											1		

T1: MgO 1 test area; T2: MgO 2 test area; T3: $Mg(OH)_2$ test area; C1: Control test area.

Figure A6 The photos of the dominant benthic organisms in this verification test.

Theora fargilis, Family: Semelidae, Class: Bivalvia, Phylum: Mollusca

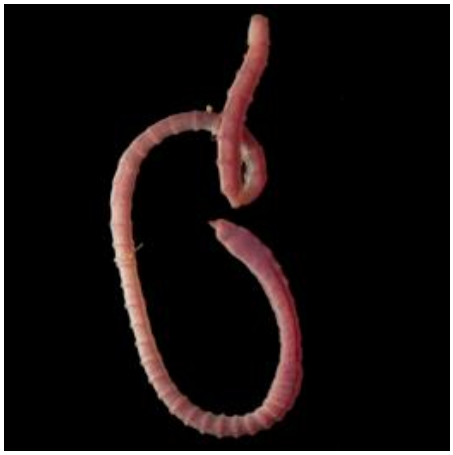
Number of individuals in this survey: around 50 to 100 individuals

Scoletoma sp., Family: Lumbrineridae, Class: Eunicida, Phylum: Polychaeta, Annelida

Number of individuals in this survey: around 30 to 50 individuals

Notomastus sp., Family: Capitellidae, Order: Capitellida, Class: Polychaeta, Phylum: Annelida

Number of individuals in this survey: around 20 to 30 individual.



The relationship between the sediment conditions (pH, H_2S and AVS) and the impact factors of test (seasonal changes, spraying of MgO and $Mg(OH)_2$)

The data of H_2S, AVS, pH were used as objective variables, the addition of MgO and $Mg(OH)_2$ and the date were used as explanatory variables in this linear model analysis. The analysis results are showed in Table A6. The value of r of each explanatory variable means the amount of changes compared the test area with control area. The positive value of r means the objective variable of the test area increased and was higher than control area, if it is negative that means it decreased and was lower than control area. In this analysis, the results clearly showed that spraying MgO and $Mg(OH)_2$ significantly raised the pH of sediments, meanwhile significantly reduced the generation of H_2S and AVS. Season changed from autumn to winter also led to a significant decrease in pH and AVS.

Table A6 Linear Model Analysis for H_2S, AVS and pH

lm (formula = H2S, AVS, pH ~ Group + DATE)						
	H_2S		AVS		pH	
	r	Pr (>|t|)	r	Pr (>|t|)	r	Pr (>|t|)
Mg(OH)2	-0.009	3.90E-04***	-0.405	1.78E-11***	0.459	2.96E-09***
MgO 1	-0.007	2.93E-03**	-0.278	1.03E-06***	0.510	9.91E-11***
MgO 2	-0.008	9.47E-04***	-0.456	1.78E-13***	0.750	2.00E-16***
22-Jun	0.022	2.49E-08***	0.096	2.30E-01	-0.231	3.08E-02*
7-Jul	0.011	2.48E-03**	0.261	1.51E-03**	0.853	1.72E-12***
21-Jul	0.012	8.77E-04***	0.285	5.53E-04***	0.217	4.24E-02*
7-Aug	0.0101	2.86E-03**	0.404	2.01E-06***	0.020	8.50E-01
19-Aug	0.014	1.92E-04***	0.006	9.44E-01	0.173	1.05E-01
3-Sep	0.0111	2.48E-03**	0.021	7.95E-01	0.007	9.50E-01
16-Sep	0.011	3.54E-03**	-0.295	3.65E-04***	0.015	8.87E-01
4-Dec	0.005	2.02E-01	-0.201	1.35E-02*	-0.217	4.24E-02*
p-value	5.89E-07***		2.20E-16***		2.20E-16***	

The explanatory variables: Group and DATE; the objective variables: H_2S, AVS and pH. Group: $Mg(OH)_2$, MgO 1 and MgO 2; DATE: 22-Jun, 7-Jul, 21-Jul, 7-Aug, 19-Aug, 3-Sep, 16-Sep, 4-Dec; p-value: p-value of F-Statistic; Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

The relationship between the impact factors of test (seasonal changes, spraying of MgO and $Mg(OH)_2$) and the benthic organisms (species and individuals)

The total number of species and individual of each test area and control area were used as objective variables, the spraying of MgO and $Mg(OH)_2$, the pH of sediments and the date were used as explanatory variables. The results of linear model analysis were showed in Table A7. As

the results showed, it is clearly and statistically confirmed that the increase in pH by spraying of MgO and $Mg(OH)_2$, that is, the weak alkaline of sediments is effective for increasing the number of species and individual of benthic organisms.

Table A7 Linear Model Analysis for Species and Individuals

lm (formula = Species , Individual ~ pH + DATE)				
	Species		Individual	
	r	Pr (>|t|)	r	Pr (>|t|)
pH	9.841	1.73E-09***	59.931	6.09E-09***
22-Jun	16.022	4.16E-15***	175.834	2.00E-16***
21-Jul	9.868	3.01E-08***	120.765	2.20E-16***
19-Aug	5.552	6.87E-04***	137.912	2.20E-16***
16-Sep	-1.648	2.88E-01	25.351	1.20E-02*
4-Dec	6.632	7.48E-05***	36.985	4.44E-04***
p-value	2.20E-16***		2.20E-16***	

lm (formula = Species, Individual ~ Group + DATE)				
	Species		Individual	
	r	Pr (>|t|)	r	Pr (>|t|)
Mg(OH)2	7.5	1.94E-09***	46.333	2.25E-07***
MgO 1	7.5	1.94E-09***	36.667	2.12E-05***
MgO 2	9.667	5.70E-13***	48.167	9.17E-08***
22-Jun	13.75	1.88E-15***	162	2.00E-16***
21-Jul	12	3.50E-13***	133.75	2.00E-16***
19-Aug	7.25	6.55E-07***	148.25	2.00E-16***
16-Sep	-1.5	2.57E-01	26.25	9.22E-03**
4-Dec	4.5	1.06E-03**	24	1.68E-02*
p-value	2.20E-16***		2.20E-16***	

The explanatory variables: Group, pH and DATE; the objective variables: Species and Individuals. Group: $Mg(OH)_2$, MgO 1 and MgO 2; DATE: 22-Jun, 7-Jul, 21-Jul, 19-Aug, 16-Sep, 4-Dec; p-value: p-value of F-Statistic; Statistical significant level as follows: (***) $0<P<0.001$, (**) $0.001<P<0.01$, (*) $0.01<P<0.05$.

The relationship between the pH of sediment and the benthic organisms (dominant phylum)

Mollusca, Annelida and Echinodermata are three dominant phylum detected in this verification test. In this analysis, pH were used as explanatory variables, the species and individual of three dominant phyla were used as objective variables. Results of this linear model analysis were showed in Table A8. The results also confirmed that the increase in pH by spraying of MgO and $Mg(OH)_2$ is effective for increasing the number of species and individual of three

dominant benthic organisms.

Table A8 Linear Model Analysis for Species and Individuals of Three Dominant Phyla

lm (formula = Species ~ pH)			
Phylum	pH		p-value
	r	Pr (>|t|)	
Mollusca	4.583	<0.001***	<0.001***
Annelida	3.134	0.040*	0.001***
Echinodermata	0.853	0.002**	0.002**

lm (formula = Individual ~ pH)			
Phylum	pH		p-value
	r	Pr (>|t|)	
Mollusca	63.66	<0.001***	<0.001***
Annelida	25.81	0.020*	<0.001***
Echinodermata	5.428	<0.001***	<0.001***

The explanatory variables: pH; the objective variables: Species and Individuals. p-value: p-value of F-Statistic; Statistical significant level as follows: (***) 0<*P*<0.001, (**) 0.001<*P*<0.01, (*) 0.01<*P*<0.05.

Conclusion

In this verification test of ETV program, the pH of sediments could be maintained at around 8.0 or more by spraying MgO and $Mg(OH)_2$, and the suppression effect on generation of H_2S and AVS were statistically confirmed by using linear model analysis methods. In addition, it was also clearly and statistically clarified that the weak alkaline could effectively increase the number of species and individuals of benthic organisms. Moreover, the survival and increase of benthic organisms accelerated the benthic activities and the effectiveness of bio-turbation that improved the purification effect on sediments.]

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