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The effect of recycled waste oyster shell powder applied to organically enriched marine sediment as oxygen releasing compound

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Eutrophic influx, being accumulated in marine sediments and its releases into the overlying water body are very important in most of the coastal areas in Korea. Nitrogen and sulfur are regarded as the most attentive elements in the metabolism of the marine ecosystem. The two groups of contaminants could be transformed into corresponding reduced compounds inside the sediment environment and its release into ambient water from sediments may have a significant impact on water quality resulting in increased eutrophication. In order to evaluate the remediation ability of ultrasound treated oyster shell powder (OSP) and raw oyster shell powder (OSP) applied to organically enriched sediments in terms of suppressing nutrient flux and acid volatile sulfides (AVS). The ultrasound treated OSP was found to be oxygenated to rather peroxide as oxygen releasing compound. The application of treated OSP decreased the concentrations of ammonium nitrogen, acid volatile sulfide, and chemical oxygen demand, whilst it increased dissolved oxygen, sulfate, and nitrate concentration significantly in the overlying water compared to the raw OSP applied basin with control. The treated OSP was successfully tested as a controlled oxygen releasing compounds (ORC) in the organically enriched sediment to reduce eutrophication. Denaturing gradient gel electrophoresis (DGGE) and community phylogenetic affiliation analyses revealed that nitrifying/denitrifying bacteria and sulfur-mediating bacteria were positively involved in the simulation experiments. It should be noted that *α*- and *β*- *Proteobacteria* (sulfur-oxidizing bacteria) were commonly identified in the microflora of sediment applied with the oxygenated oyster powder.

[**Keywords**: Bacterial community, Bioremediation, Eutrophication, Marine sediment, Ultrasound treated oyster shell]

Introduction

In the world, human population is increasing day by day and hence increased attention is being focused on natural resources and the marine environment associated with limited resources, worldwide[1,2]. Recently, marine eutrophication has been accelerated due to industrial wastewater discharge, agriculture releases, a large number of aquaculture activities (e.g. oyster and fish farms), and through human activities to be the most common but serious environmental issues[3-5]. In consequence of input nutrients containing organic matter, nitrogen and phosphorus are being deposited in the sediments until algal bloom in the marine environment[6]. These nutrients could deposit into the bottom sediment and it can be released back into the overlying water[7]. Most often increase in nutrient load in marine sediments and water is the cause of eutrophication. This eutrophication matter indicates that the abundant nutrient input overwhelms the capability of water bodies to self-purify, as a result leads to increased algal blooms and changes in ecosystem characteristics[8,9]. Sediments are important sources of nutrients to aquatic ecosystems[10]. The organic matter and nutrients involved in the sediment can be decomposed by aerobic microbial activities. However, occasional abruption of oxygen supply can cause the hypoxic condition in the sediment and subsequently produce toxic byproducts such as NH_4^+ and H_2S releasing these into the overlying water column.

Nitrogen is a primary nutrient, playing a significant role in the aquatic ecosystem[11,12]. Nitrogen transformation and its recycling in coastal sediments contribute significantly to eutrophication in the coastal areas as major nutrients for aquatic life. The O_2 consumption in the sediments may be contributing

to release of nitrogen from sediment to water, which is the chief internal source of coastal water polluion[13]. The nitrogen concentration in the contaminated sediments, which is being released into the overlying waters of oceanic environments, is seemed like a major element of the internal source. Consequently to control eutrophication, it is important to alleviate nutrients release flux from sediments as well as diminish nutrients load.

In the aquatic environment, sulfate (SO_4^{2-}) is the most plentiful and thermodynamically stable form of sulfur due to a great variety of sulfate-reducing bacteria (SRB)[14]. The sulfur cycle is closely linked to nitrogen cycles. Microorganisms acting significant part in transformations of sulfur-like metabolic redox reactions such as dissimilatory sulfate reduction by SRB and sulfide oxidation by chemolithotrophic sulfur bacteria[15]. The anaerobic microorganisms are sulfate-reducing bacteria, inhabiting anoxic sediments, and as a result generates sulfide from the sediments. Availability of acid volatile sulfide indicates the potential for H_2S generation, possibly leading to malicious, anaerobic habitats for the marine ecosystem.

A great deal of previous work has been done on applications of oxygen releasing compounds (ORCs) such as MgO, MgO_2, $Mg(OH)_2$, CaO, CaO_2, etc. and in parallel, application of chemical and/or microbial agents for augmenting bioremediation[16-18]. Treated oyster shell powder is gradually released of oxygen when decomposing in contact with water. The released O_2 can promote *in-situ* aerobic microbial degradation and enhance contaminant's oxidation[19-21]. These materials can alleviate the anaerobic condition and raise up aerobic condition in the bottom sediment to augment the aerobic bacterial action. For bioremediation in the sediment and water, oxygenation is a crucial factor[22,23]. Therefore, it is essential to understand how oyster shell powder may affect the complex nitrogenous transformations with dominant sulfur activity in the marine system and their contributions in nitrogenous nutrients.

The objectives of this work are summed up to (1) examine the remediation efficiency of raw and ultrasound treated oyster shell powder for suppressing nutrients flux of organically enriched sediments; (2) investigate sulfur & nitrogen dynamics behavior; and (3) identify microbial activities in the sediment *via* genetic analysis.

Materials and Methods

Study area and sampling

The samples (marine water and sediment) were collected from in the vicinity of marine college, Tongyeong city (34°50′28.5″ N and 128°28′16.4″ E), in the southeast coast of South Korea. The consistent pollution from waste dumping, fishing and aquaculture activities and its malicious odor was the common phenomenon observed throughout the sampling area. The sediment samples collection was performed by standard procedure where the sediments were collected from 10 m below the water level with the help of stainless-steel grab sampler at a depth of 0-20 cm. The seawater samples were collected at the same contaminated site at a depth of 0.5 to 1 m below the water surface. The sampling was confined in a relatively small area to maintain uniformity in sampling. The sampled sediments were screened and homogenized for the removal of plant garbage and pebbles and for better quantification of its physico-chemical properties as well.

Functional oyster shell powder preparation by ultrasound treatment

The waste oyster shells comprise 92.08 % of calcium carbonate ($CaCO_3$)[24]. Oyster shells were dried in a drying oven at 80 °C for 12 h. The dried oyster shells were crushed and fine powder was passed through the 100-200 mesh screens. Then, the waste shell powder was sonicated with distilled water for 60 min (powder: water (w/w) = 1:2) using an ultrasonicator (T18, Ultra-turrax, USA). The dried powder was milled and then oxidized with hydrogen peroxide. The re-milled powder was then used for the experiments, which is called functional oyster shell powder, as oxygen releasing compound[25].

Basin incubation

In the laboratory setup, 300 g of sediment sample was prepared and it was stirred to be fully homogenized in a 2 L graduated basin. In the experimental basin, 300 g of sediment and 1.5 g (0.5 % w/w) treated OSP and raw OSP were mixed, where 300 g of sediment without OSP was used as control. Thereafter, 1 L of filtered seawater was slowly added to the basin to avoid disturbing the sediments. Then all the basins were enclosed by aluminum foil and were kept in the incubator at a fixed temperature. During the experimental period, all basins were conditioned at 22 °C under light and dark in alternative 12 hours. The test was conducted at 0, 5, 10, 15 and 20 days.

Analysis of physico-chemical characteristics

The water and sediments were separated from the column and the sediments were homogenized. The pH and oxidation-reduction potential (ORP) were determined by using a multifunctional meter (Orion 3 star, USA). Dissolved oxygen of the overlying water was measured with a DO meter (YSI 550A, USA).

Chemical analysis

The sediment sample was centrifuged (200 rpm for 20 min) to collect the supernatant pore water. The overlying water and supernatant were filtered by a glass microfiber membrane filter (GF/C, Whatman, UK). The iodometric titration method was performed to determine the chemical oxygen demand (COD) with the aid of potassium permanganate as an oxidizing agent.

On the other hand, the turbidimetric method was used to determine the sulfate content. The concentration of T-N, NO_3^--N, NO_2^- -N, and NH_4^+-N in the filtrate were analyzed by potassium persulfate oxidation assays, cadmium-copper column reduction method, N-(1-naphthyl)-ethylenediamine adsorption spectrophotometry, and indophenol blue method respectively, according to the previously described methods[26]. The AVS concentration of the sediment was determined by the sulfide detection tube (detector Tube No. 201H; measuring range 0.02-0.20 mg, GASTEC, Japan). The spectroscopic investigations were done by UV Mini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). For all experiments, overlying water and sediments were analyzed in triplicate and average results were used.

Analysis of microbial community

Total DNA was extracted from the sediment samples using a FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) following the protocol provided by the manufacturer as described by Rickard & Morse[15]. Firstly, 16S rDNA was extracted from the sediment samples and were PCR amplified using primers 27f and 1492r. The bacterial V3 region of 16S rDNA genes was amplified using primer GC-341F and 518r equipped with 40-bp GC-clamps (Bioneer Corporation, South Korea). The qPCR amplification thermocycling condition was as follows: initially denatured at 95 °C for 2 min, followed by 35 cycles of 95 °C for 20 sec, 48 °C for 45 sec, and 72 °C for 1 min and then an elongation at 72 °C for 10 min. The products of amplified PCR were evaluated on 1 % (w/v) of agarose gels using electrophoresis. Subsequently, the obtained 16S rDNA gene sequence was subjected to BLAST searching in the National Center for Biotechnology Information (NCBI).

Results and Discussion

Change of dissolved oxygen concentration in overlying water

Dissolved oxygen (DO) concentration with time is shown in Figure 1. The ultrasound treated oyster powder applied basin overlying water dissolved oxygen concentration was observed to be higher than other basins owing to its oxygen releasing capacity indicating its peroxide portion added by sonic energy. On the other hand, the application of raw shell powder containing a lot of surface pores made the level of dissolved oxygen lower than any other cases since its inherent adsorbing ability is well known. It was found that prolonged shelf life of the peroxide ensured a slow, somewhat controlled release of molecular oxygen out of the ORC molecules at least for 20 days long although a rise-and-back of DO in control basin was not so explanatory.

Change of sediments chemical oxygen demand (COD)

COD is an important index of polluted water, expressing the chemical oxidation of involved organic matter. COD represents all the matter including organic compounds inhibiting biodegradation. Hence, it became necessary to check the concentration of COD in with and without oyster powders (Fig. 2). In the control basin, found a natural attenuation of COD in short times as well as that of ultrasound treated

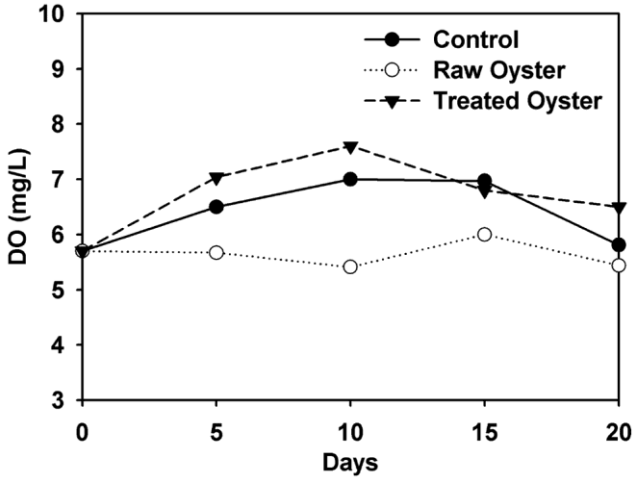

Fig. 1 — Change of DO in the overlying water with addition of raw and treated oyster shell powder (0.5 % w/w) treatment with control for 20 days

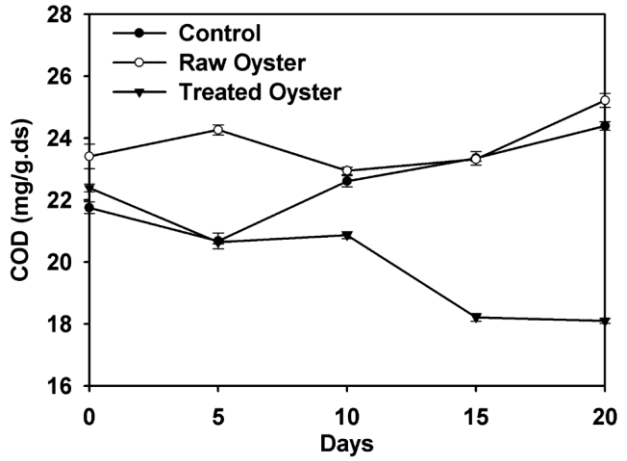

Fig. 2 — Change of COD in the sediment with addition of raw and treated oyster shell powder (0.5 % w/w) treatment with control for 20 days. Bar represents standard deviation of triplicates

shell powder basin. It might be caused by certain microbial activity in those aerobic surroundings. Soon it seemed to be offset by the continual release of nutrients out of the sediments. However, in the treated basin, COD kept decreasing overtime till 18 mg/g or so, eventually showing enhanced microbial activity with excess oxygen supply by the ultrasound-treated powder[16]. As a result, the performance of treated oyster powder was more efficient than that of raw oyster powder in COD removal from the sediments. In contrast, the concentration of COD in the sediment remained more or less constant in the raw oyster powder treated basin because the raw powder would not produce extra oxygen even in contact with water, and its small pores may not provide with sufficient surface area to scavenge organic molecules in the bulky water-phase.

Effect of OSP treatment on nitrogen concentration in the sediment and water

Nitrogen is an essential and abundant element for aquatic life in the oceanic environment, and transforms into many forms like N_2, NO_2^-, NO_3^-, NH_4^+ and other nitrogenous organic matter in its cycle along with other elements such as phosphorus, sulfur, iron, and carbon. The nitrogenous compounds in the sea environment can be influenced by factors like the types of sediment, sedimentation rate, volume and types of organic matter, redox condition, and the intensity of mineralization of organic matter[27]. The characteristics of nitrogen concentrations such as NO_3^--N, NO_2^- -N, and NH_4^+-N have been examined during the experimental period. Raw and treated

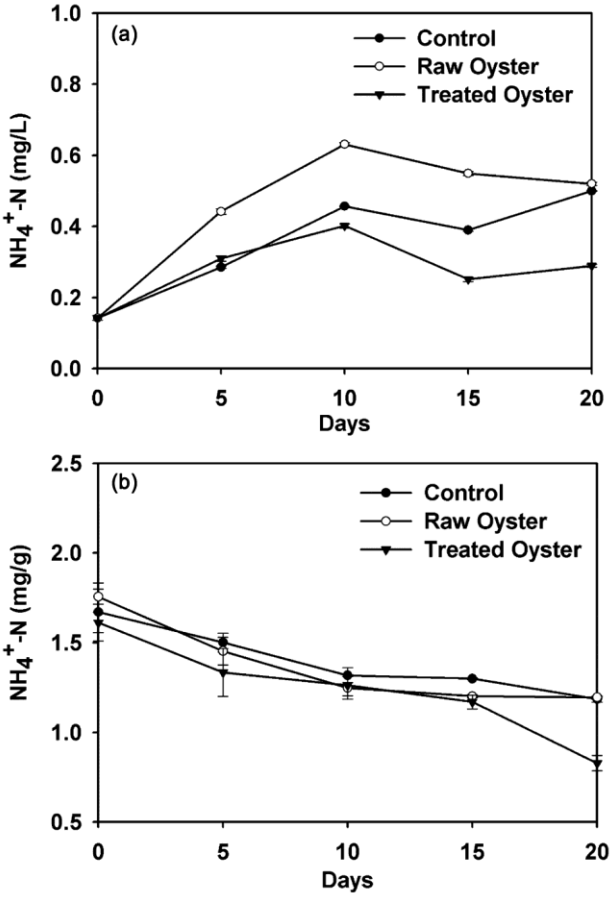

Fig. 3 — Concentration of NH_4^+-N in the overlying water (A); and sediment (B): control, raw oyster and treated oyster shell powder (0.5 % w/w) treatment for 20 days. Error bar represents standard deviation of triplicates

oyster shell powders were used at 0.5 % (w/w) per basin except in control basin as described earlier. The NH_4^+-N and NO_3^--N concentrations in the sediments and overlying water are shown in Figures 3 and 4, respectively. At the beginning of the experiment (day 0), NH_4^+-N concentrations of the sediment and water were differentiated, e.g., 1.6~1.8 mg/g and 00.14 mg/L, respectively. That is, there was about ten times higher NH_4^+-N concentration in the sediments. NH_4^+-N concentration in the sediment decreased almost linearly with time in both treated and control basins. From a view of material balance over the system, ammonium-N would be transformed or released away. Another reason why the ammonium has been diminished in the control and raw oyster applied basins could be self-disappearance through a much complicated, but very feasible denitrification process in the anaerobic environment. In longer times (15 days or more), found an additional decrease in

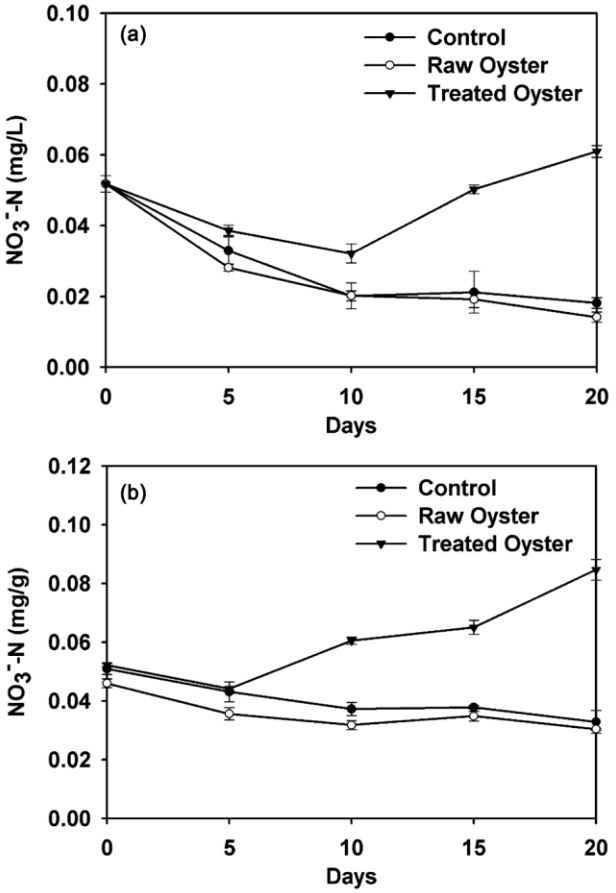

Fig. 4 — Concentration of NO_3^--N in the overlying water (A); and sediment (B): control, raw oyster and treated oyster shell powder (0.5 % w/w) treatment for 20 days. Error bar represents standard deviation of triplicates

ammonium-N in the sediment of the treated basin. It strongly implies that oxygen availability from the peroxide compound ought to inspire a microbial growth. The concentration of NH_4^+-N in the water increased for the first 10 days and then reduced or leveled-off in the treated oyster and control basins.

Noticeable drops of NH_4^+-N concentrations in the second half is may be due to the on-going oxidation or evaporation of ammonium gas which depends on the local environmental conditions such as temperature and pH. Meanwhile, a significant rise of ammonium concentration was found in the raw oyster powder basin; whilst there was a slower increase in NH_4^+-N in the other basins. Anaerobic condition and higher pH in the sediments of the raw oyster basin might have facilitated the release of ammonium out of sediment pores while the less anaerobic or more hypoxic environment for treated oyster basin could have reduced ammonium release by inducing its conversion to a more oxidized form(s). Enough oxygen content indicated that nitrifying microbes could be populated to precede the ammonia oxidation. Bacterial activity is described in detail in the subsequent text of this article. Ammonium nitrogen concentration measured over time was significantly lower in the treated oyster shell powder applied basin than any other basin, thus indicates better efficacy of ultrasound treated oyster shell powder over remediation of anaerobic coastal sediments.

NO_3^--N is the final form of NH_4^+-N, if oxidized. The time change of nitrate concentration was monitored in Figure 4. NO_3-N concentrations in the two layers in the raw oyster treated and control basins remained nearly constant for the full incubation period, whereas that in the treated oyster basin kept soaring till the end of the experiment. The patterns were similar in both graphs, which suggests an oxidation of ammonium-N in one end and a sort of denitrification in the other end (nitrate to gaseous nitrogen), implying that the ultrasound treated oyster shell power worked well as ORC. Comparison of nitrate curves in Figures 4A and 4B logically supports the change of the anaerobic condition to the oxic state, accompanied by nitrogen-mediated bacterial activity and subsequent release of nutrients towards the bulk of water from sediments. Most of the total nitrogen (NH_4^+, NO_3^- and NO_2^-) were in NH_4^+ in the sediment and water. The NO_2^--N concentration was very low and was negligible in the experiment[28]. Accordingly, the total nitrogen (T-N) behavior was regarded similar to that of NH_4^+ in both water layers (graph not shown).

The concentration of NO_3^--N was highly correlated with COD in the sediment. NO_3^--N concentration was found to increase whereas the concentration of COD decreased in the treated oyster shell powder applied basin. In addition, increased COD correlates well with decreased NO_3^--N concentration in the raw oyster treated basin and with control. This counter-correlation strongly implies microbial activation in the presence of oxygen. Some nitrifying and denitrifying bacterial species were found through DGGE analysis (Table 1). In the treated oyster shell powder applied basin, the electrophoretic bands showed the existence of groups like *Nitrosomonas* species which might perform the oxidation of ammonium to nitrite, and *Nitrobacter* that could involve in the oxidation of NO_2^- and in the increase of NO_3^- concentration. In the control and raw oyster applied basins, *Pseudomonas* and *Halomonas* species

Table 1 — Base sequences of 16S rDNA read from DGGE bands

DGGE band	Description	NCBI accession no.	Identity (%)
1	*Desulfuromonas* sp.	DQ395053.1	98 %
2	*Halomonas* sp.	JX310218.1	98 %
3	*Klebsiella* sp.	MK587663.1	96 %
4	*Desulfuromonadaceae* bacterium	AM501590.1	99 %
5	*Pseudomonas* sp.	LC002926.1	98 %
6	*Nitosomonus* sp.	JF506026.1	99 %
7	*Thiobacillus* sp.	KU519595.1	95 %
8	*Bacillus* sp.	KR095405.1	100 %
9	*Sulfuriferula* sp.	NR_114805.1	97 %

DGGE: denaturing gradient gel electrophoresis, NCBI: National Centre for Biotechnology Information

might have performed NO_3^- reduction in the sediments. Remembering that the NH_4^+-N concentration in the overlying water of treated oyster powder applied basin was found to be lower than any other basin (Figure 3A) and is might be due to the reduction of ammonium release from sediment to water. The pore water NH_4^+-N concentration was decreased dramatically after the application of treated oyster powder. Consequently, the significant decrease of ammonium concentration seems to be brought by the formation of NO_3^- in the treated oyster powder applied basin.

Change of acid volatile sulfide (AVS) and sulfate (SO_4^{2-}) concentration in the sediment

Sulfur (S) is ubiquitously distributed in the oceanic environment and exists in various forms such as: elemental sulfur (S_8), sulfides (S^{2-}), sulfite (SO_3^{2-}), polysulfides (S_n^{2-}), sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), metal sulfide, and in various organic sulfur compounds. Sulfate (SO_4^{2-}) is a dominant species and is thermodynamically stable. Under the oxic condition, sulfides may be oxidized to sulfate and the anoxic condition drives sulfate to be reduced to sulfide. Sulfides are known to be too toxic, corrosive, and malodorous to aquatic organisms. In the marine environment, sulfides are generated predominantly by sulfate reduction[29]. It is more comprehensive to quantify acid volatile sulfide (e.g. H_2S and FeS; commonly AVS), a representing form of reduced sulfur produced by anaerobic sulfate reduction.

Noting the fact that sulfate is rather a dominant compound observed in current experiments (Figure 5). In the control experiment, more than 10.8 mM of sulfates availed initially and were then diminished slightly to 8.4 mM. As the local environment became more anoxic, sulfates were reduced and acid volatile sulfides were produced. Sulfate in the raw oyster basin also decreased possibly due to reduction of sulfur and adsorption capacity of raw oyster powder.

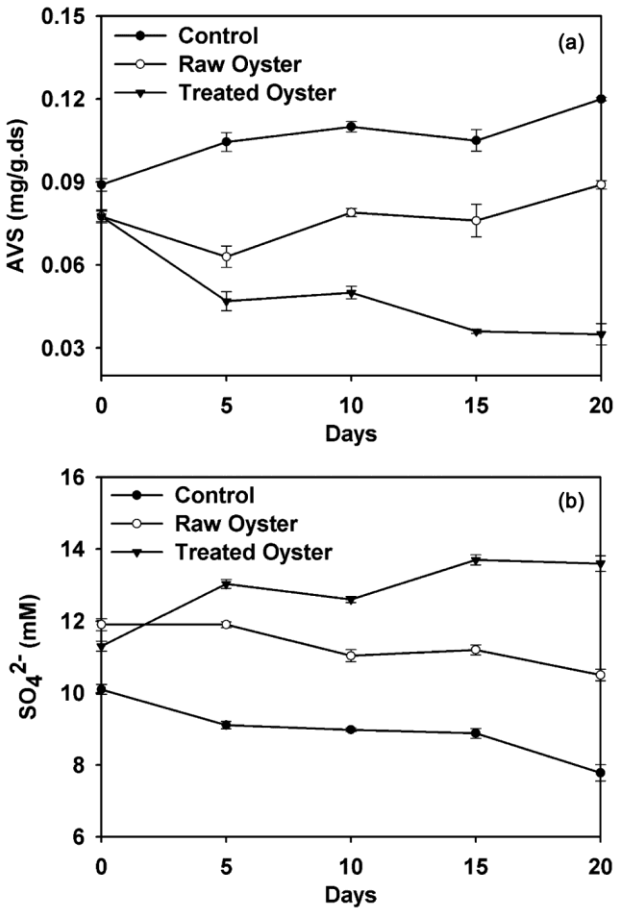

Fig. 5 — Change in AVS (A); and sulfate concentration (B): control, raw oyster and treated oyster shell powder (0.5 % w/w) treatment for 20 days. Error bar represents standard deviation of triplicates

Its reduced form is shown in Figure 5A as an increase of sulfides. Sulfate reductive reactions may enable this direct transformation within the pore water phase which is a reasonably limited space: Equations (1) and (2). It is interesting to find higher sulfate and lower AVS with time in the ultrasound treated oyster basin unlike the other basins because for the sulfur,

presence of oxygen would directly facilitate oxidation reactions with an aid of living oxidizers. Almost half of AVS molecules decomposed in 20 days. Initial oxidation-reduction potential (ORP) in the sediment was very low (-215.3 mV), which confirmed the anoxic condition in all basins.

$$CH_2O + \frac{1}{2}SO_4^{2-} + H^+ \rightarrow \frac{1}{2}H_2S + CO_2 + H_2O \quad \ldots (1)$$

$$SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O \quad \ldots (2)$$

Microorganisms are involved in the biogeochemical transformation of sulfur compounds in the sediment. Generally, anoxic sediments comprise of active sulfate-reducing bacteria[30]. Therefore, in the control and raw oyster treated basins, reduction of SO_4^{2-} from sediment pore water may produce various protolytic species such as H_2S and NH_3. In the presence of sulfides, NO_3^--N reduction can befall if NO_3^- acts as an electron acceptor and sulfide as the electron donor in the marine sediments[31]. The increased sulfide concentration enhanced the rate of reduction of SO_4^{2-} and NO_3^--N in the control column rather than in the treated oyster shell powder applied basin. In the treated oyster powder basin, it has been observed that the bacterial species such as *Thiobacillus* belonging to the chemolithotrophic *β-Proteobacterium*, which is supposed to oxidize sulfides in the sediment where NO_3^- accumulates to play as an electron acceptor in the presence of O_2 and/or peroxides. Stolp[32] also summarized the microbial community around the anoxic environment: availability of H_2S and O_2 allows sulfur-oxidizing bacteria like *Thiobacillus*, *Thiomicrospira*, *Sulfolobus*, and *Sulfuriferula* whereas a large group of bacteria such as *Thioploca*, *Beggiatoa*, *Klebsiella*, and *Aeromonas* actively converts abundant SO_4^{2-} to H_2S in sulfur cycle of marine areas. Sulfate reducers belonging to *δ- Proteobacteria* were also found in the control sediments (Table 1).

Depletion of oxygen in the reduced sediment may trigger denitrification where NO_3^- is reduced back to NH_3, in lieu of N_2 gas by dissimilatory nitrate reduction to ammonia (DNRA) empowered by the prevailing H_2S[33]. Consequently, the higher concentration of sulfides was induced in the control and raw oyster treated sediments that would inhibit nitrification and/or facilitate denitrification, but the lower concentration of AVS in the treated oyster basin, depends on the existence of oxygen or oxygen carriers.

Analysis of DGGE and phylogenetic affiliation over bacterial community

Verification of microbial consortium is essential for bioremediation in the sediments. Sediment harbors a complicated ecosystem for microbes, and they are predominantly affiliated with phyla *Proteobacteria*, *Actinobacteria*, *Chloroflexi planctomycetes*, and *Bacteriodetes*[34-36]. DNA bands were obtained from a denaturing gradient gel electrophoresis (DGGE) analysis for sediment samples using specific primers (Fig. 6). A total of 9 bacterial bands were identified

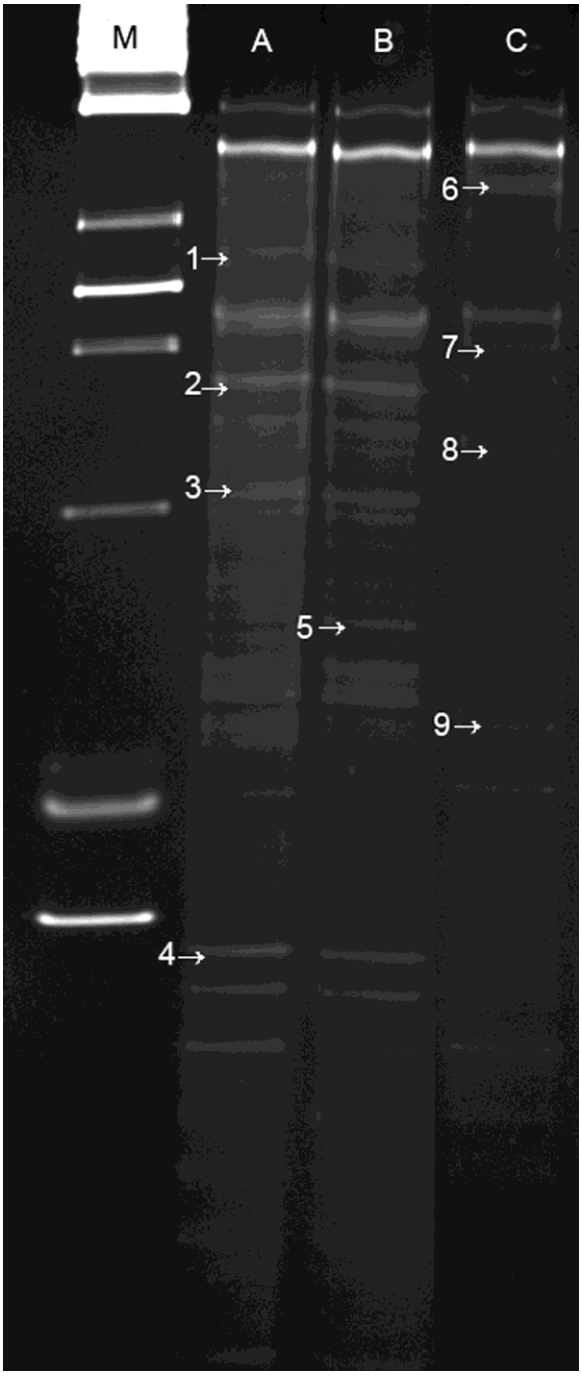

Fig. 6 — DGGE profiles of PCR-amplified 16S rDNA genes of bacteria: control (A), raw oyster applied sediment (B), and treated oyster applied sediment (C) after 20 days

and all members of *Proteobacteria* (*α-, β-, γ-, δ-Proteobacteria*) group were detected. The overall population showed DNA combinations of the inhabitant microbial community in accordance with the raw oyster and treated oyster powder treatments.

Using DGGE band profile, the location and limpidity of all bands were checked in a number of PCA analysis; A (control), B (raw oyster powder), and C (ultrasound treated oyster powder). The BLAST (Genebank) results for 16S rDNA sequence of the main bands identified: members of the *α-, β- Proteobacteria* (sulfur-oxidizing bacteria) i.e., genera *Nitrosomonas*, *Thiobacillus*, *Bacillus*, and *Sulfuriferula* in the ultrasound treated oyster basin sediments; *γ-, δ- Proteobacteria* (the most often illustrated lineage among sulfate-reducing bacteria) i.e., genera *Desulfuromonas*, *Halomonas*, *Desulfuromonadaceae*, *Pseudomonas*, *Klebsiella*, etc. in the raw oyster treated basin with control (Table 1).

Conclusions

The present study investigated nitrogen, sulfur and carbon circulations along with verification of related bacterial community in the simulated coastal sediments *via* biochemical analysis and DNA identifying methods. Our focus was on the effect of oyster powder treated with sonic energy over the simulated sediment/water system. The results showed that the ORC material has clearly ensured further oxidation of reduced nitrogen and sulfur compounds by increasing DO, thus relieving out fluxes of those compounds as well as diminishing their concentrations. Additionally, it was also capable of oxidizing the reduced N- and S- compounds (ammonium-N and AVS) in the bulk phase for 3 weeks, a relatively long time period. Considerable reduction of NH_4^+-N and AVS and increase of corresponding oxides are likely to prove the existence of nitrifying and sulfur-oxidizing microbial species whereas diminishment of reductive compounds in the anoxic state may confirm a microbial activity provoked by denitrifying and sulfur-reducing bacteria.

Overall, oyster shell powder showed its great potential to physical and biochemical remediation in organically enriched coastal sediments, and the ultrasound treated oyster powder was found to effectively catalyze attenuation of eutrophication.

Acknowledgments

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

The first author MAK was involved in the conceptualization, methodology, sampling, analysis and writing original draft. BGK helped in sample analysis process. DC: writing review and editing the article. The entire research work was supervised thoroughly by SHK.

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