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Eco-friendly and efficient *in situ* restoration of the constructed sea stream by bioaugmentation of a microbial consortium

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복합미생물 생물증강법을 이용한 인공해수하천의 친환경 효율적 현장 수질정화

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A constructed sea stream in Yeongdo, Busan, Republic of Korea is mostly static due to the lifted stream bed and tidal characters, and receives domestic wastewater nearby, causing a consistent odor production and water quality degradation. Bioaugmentation of a microbial consortium was proposed as an effective and economical restoration technology to restore the polluted stream. The microbial consortium activated on site was augmented on a periodic basis (7~10 days) into the most polluted site (Site 2) which was chosen considering the pollution level and tidal movement. Physicochemical parameters of water qualities were monitored including pH, temperature, DO, ORP, SS, COD, T-N, and T-P. COD and microbial community analyses of the sediments were also performed. A significant reduction in SS, COD, T-N, and COD (sediment) at Site 2 occurred showing their removal rates 51%, 58% and 27% and 35%, respectively, in 13 months while T-P increased by 47%. In most of the test sites, population densities of sulfate reducing bacterial (SRB) groups (Desulfobacteraceae_uc_s, Desulfobacterales_

uc_s, Desulfuromonadaceae_uc_s, Desulfuromonas_gl_uc, and *Desulfobacter postgatei*) and Anaerolineales was observed to generally decrease after the bioaugmentation while those of Gamma-proteobacteria (NOR5-6B_s and NOR5-6A_s), Bacteroidales_uc_s, and Flavobacteriales_uc_s appeared to generally increase. Aerobic microbial communities (Flavobacteriaceae_uc_s) were dominant in St. 4 that showed the highest level of DO and least level of COD. These microbial communities could be used as an indicator organism to monitor the restoration process. The alpha diversity indices (OTUs, Chao1, and Shannon) of microbial communities generally decreased after the augmentation. Fast uniFrac analysis of all the samples of different sites and dates showed that there was a similarity in the microbial community structures regardless of samples as the augmentation advanced in comparison with before- and early bioaugmentation event, indicating occurrence of changing of the indigenous microbial community structures. It was concluded that the bioaugmentation could improve the polluted water quality and simultaneously change the microbial community structures via their niche changes. This *in situ* remediation technology will

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contribute to an eco-friendly and economically cleaning up of polluted streams of brine water and freshwater.

Keywords: Flavobacteriaceae, bioaugmentation, microbial consortium, polluted stream, pyrosequencing, restoration, sulfate reducing bacteria (SRB)

Rapid population growth and urbanization deteriorate the qualities of water, soil and air. A serious pollution of the aquatic environments poses a significant threat to human health as well as the natural ecosystem disruption. Recently tangible and intangible values, and economic value of the stream ecosystems have become a hot issue in view of the environmental preservation. A recent study estimates the values of freshwater ecosystem services of the five major river watersheds in South Korea reach \$435 million, \$356 million, \$210 million per year in terms of water supply, water purification, and natural disaster control, respectively while the total government expenditure to improve freshwater ecosystem services reached \$7.7 billion in 2013 (Ahn and Kim, 2016).

An eco-friendly stream remediation technology was first introduced since the year 2000 (Kim and Yeo, 2004), and then a multi-level type of wetland remediation system more effective in land use and nitrogen removal (Jung and Lin, 2004) and a hybrid type of wetland remediation system for physical and biological treatments of polluted streams (Kim *et al.*, 2006) were investigated. A rope media filter was applied to improve stream water quality but removal rates of nitrogen and phosphorus were in the range of 15~36% (Chung and Im, 2006). More recently improvement of water quality of secondarily treated domestic wastewater utilizing a natural stream restoration technology was performed (Ahn *et al.*, 2009), and an active carbon biofilter system was employed to clean up the static region of a polluted stream (Oh *et al.*, 2010).

Bioaugmentation is one of the bioremediation technologies where addition of selected microbial strains/mixed cultures of specific functions is made to effectively remove target pollutants in various environmental settings such as wastewater treatment plant, soil/groundwater, and aquatic environment. A bioaugmentation technology utilizing a microbial agent of special function could be an alternative to restoration of the polluted small streams. Wastewater, stream water and aquarium water were

purified employing titanium bioballs carrying activities of photooxidation and microbial degradation (Yang, 2003). This treatment system may have a limitation in an effective removal of organic pollutants in the water column since UV cannot easily pass the water to reach the titanium photocatalyst. A microbial consortium product composed of various species of aerobes (*Bacillus* sp., *Rhizobium* sp., *Azospirillum* sp., *Flavobacterium* sp., *Streptomyces* sp., and *Paenibacillus* sp.) were used to improve the water quality but the aerobic bacteria may not work in anaerobic conditions (Biovankorea, 2010). Several species of photosynthetic bacteria possessing a good nitrogen removal activity (*Rhodobacter blasticus*, *Rhodobacter capsulatus*, *Rhodocyclus tenuis*, *Rhodomicrobium vannielii*, and *Rubrivivax gelatinosus*) immobilized on the ceramic media were used to treat the stream water of low concentration ammonium within a contact oxidation reactor under aeration, in which total nitrogen was removed up to 82.8% (Kim and Lee, 2010). This process, however, was not proven in its efficacy in the stream *in situ* carrying low organics and low nitrogen. A pilot scale contact oxidation ditch study for bioremediation of polluted stream water employing a bioaugmentation of nitrifying bacteria showed that removal rates of TN and NH_4^+ -N were 50.3% and 60.1%, respectively and blooms of ammonia oxidizing and nitrifying bacteria were observed (Jiao *et al.*, 2011). Recently a pilot scale combination system of micro bubble process (MBP) and attached microbial pipe system (AMPS) was applied to remediate an urban small stream (Kim *et al.*, 2013). Herein, maximal removal rates of TSS and T-P in MBP were 69.7% and 70.1%, respectively while AMPS process exercised the T-BOD₅ maximal removal rate (68.5%), indicating the system feasibility for application in stream restoration. A porous concrete bioaugmented with effective microorganisms (EM) was used to purify the stream water where good removal efficiencies of COD (62%), T-N (17%), and T-P (15%) were observed during the first 120 days but the efficiencies decreased afterwards. This posed maintenance problems including back washing or replacement of the concrete, meaning low benefit to cost (Park *et al.*, 2014). Bioaugmentation technology becomes a promising technique to solve crucial problems in wastewater treatment plants (e.g., removal of specific refractory compounds) these days (Herrero and Stuckey, 2015). A full scale application of bioaugmentation to the wastewater treatment plants has little

been reported except one case of upgrade a full-scale activated sludge system into a contact oxidation system (Ma *et al.*, 2009), in which the consortium (mainly consisting of *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, and *Micrococcus*) enriched from the activated sludge of various petrochemical WWTPs was bioaugmented to successfully treat the recalcitrant petrochemical compounds. Lately, our study team has accomplished a successful treatment of the recalcitrant tannery wastewater at a full scale through the bioaugmentation of the microbial consortium BM-S-1 (Kim *et al.*, 2014). Recently a research was accomplished to remediate polluted sea sediments using the microbial agent EM (effective microorganisms) (Ekpeghere *et al.*, 2012) in which the microbial agent turned out to be effective in removal of odors from the polluted sediments. However, this study failed to show the removal effects of COD, T-N, and T-P using EM, indicating the microbial agent had a serious limitation in bioremediation of organic pollutants in the polluted sediments. More recently, bioaugmentation of the microbial consortium (BM-2) was applied to remediate a small urban stream polluted with domestic wastewater in which significant removals of COD, NO₃-N, and malodor were observed in the middle stream (Chang *et al.*, 2015).

A sea stream has been artificially constructed in the Yeongdo Innovation Complex, Busan, South Korea to meet recreational needs of residents in the community around. This stream utilizes seawater for its waterway maintenance. The stream, however, persistently receives domestic wastewater from the neighboring residential areas, and possesses a lifted stream bed and no slope in the waterway so that stream water keeps a poor water quality and causes malodor and other environmental problems. This really causes an accumulation of organic pollutants and other suspended solids. Moreover, there were little *in situ* remediation technologies available today. Therefore, there is a need to develop an efficient remediation technology for the polluted stream that is feasible, economical and environ-

mentally compatible.

Here in this study, a beneficial microbial consortium was applied to effectively remediate the polluted sea stream in the Yeongdo Innovation Complex. It turned out that the bioaugmentation of a beneficial microbial consortium to the polluted sea stream was able to successfully remediate organic pollutants of domestic origin.

Materials and Methods

Location of test bed and sampling sites

The location of test bed and sampling sites were shown in Fig. 1 (top) and Table 1. The test bed used to be a site for coastal sediment dumping and then an artificial sea stream was constructed on the edge on the dumping site to secure an



Fig. 1. Sampling and monitoring sites at Dongsam Sea Stream in this study (top) and bioreactor and bioaugmentation view (down).

Table 1. GPS position of sampling sites at Dongsam Constructed Sea Stream, Busan, Republic of Korea

	St. 1	St. 2	Site 1	Site 2 (St. 3)	Site 3	St. 4	St. 5
Altitude	35°4'21. 72"N	35°4'20. 60"N	35°4'24. 46"N	35°4'28. 56"N	35°4'33. 95"N	35°4'41. 43"N	35°4'47. 87"N
Latitude	129°4'55. 65"E	129°4'38. 13"E	129°4'33. 98"E	129°4'31. 90"E	129°4'30. 00"E	129°4'29. 11"E	129°4'32. 71"E

amenity facility for the residents around. In order to monitor water and sediment quality before and after bioaugmentation of the beneficial microorganisms (BM-S-1) (Kim *et al.*, 2014), five sampling sites were chosen. Site 1 and Site 3 were also added to closely monitor the bioaugmentation efficacy. The St. 3 was the same site as Site 2 where the bioaugmentation was implemented.

Implementation of *in situ* bioaugmentation system on the test bed

A stock culture of BM-S-1 was prepared in advance at a manufacturing plant by growing on molasses (0.3% w/w) and salt (0.3% w⁻¹) in a bioreactor (10 ton) (Kim *et al.*, 2014). An active culture used for *in situ* bioaugmentation was then grown on molasses (0.3% w/w) dissolved in 4,000 L of tap water at an ambient temperature (23~30°C) for 3 days with 200 L of the stock being inoculated. The pH of the active culture was ranging from 4 to 4.5. Aeration was made during growth of the active culture to maintain a proper DO level (0.8~3 mg/L). The bioaugmentation was performed at the Site 2 through a plastic hose for 2~3 h and the active culture was dispersed in direction of Site 1 and Site 3 by the tidal power (speed 0.5 m/sec). The very first bioaugmentation was done Sept. 29, 2012, and subsequent bioaugmentations were performed every 7~10 days thereafter until Nov. 2, 2013 (Fig. 1, down).

Sampling of water and sediments, and analytical parameters

The sampling for water and sediment was done 19 times for the period of July. 4, 2012~Nov. 2, 2013. The water samples were taken using van Dorn water sampler and the sediment samples using van Veen grab sampler. Analytical parameters for water were pH, DO, ORP, salinity, SS, COD, T-N, T-P, and chlorophyll-a in the water column, and COD in the sediment. SS and chlorophyll-a were measured following the standard methods (American Public Health Association, 2005) while pH, DO, ORP, and salinity were measured using a multi-purpose water quality test equipment (YSI6600V2, YSI, Inc.). COD, T-N, and T-P were measured using the seawater analysis kits of Humas, Inc. such as HS-COD (Mn)-SW, HS-TN-SW, and HS-TN-TP, respectively. NH₄⁺-N, NO₃⁻-N, and PO₄³⁻-P

were also measured once for the samples of Nov. 2, 2013 to check the mass balance of N and P, in which the seawater analysis kits of the same vender such as HS-NH₃(N)-SW, HS-NO₃(N)-SW, and HS-PO₄(P)-SW, were used, respectively.

Removal efficiencies for COD, T-N, and T-P in water column and COD of sediment were calculated based on the equation:

$$\frac{\text{Data before bioaugmentation} - \text{Data after bioaugmentation}}{\text{Data before bioaugmentation}} \times 100 (\%)$$

where data before bioaugmentation were the average value of data July 4, 2012 through August 30, 2012 and the data after bioaugmentation were the average value of data at November 2, 2013.

All the data collected in duplicate were statistically analyzed and fitted using SigmaPlot Version 12.3. Non-metric multidimensional scaling (NMDS) ordination of the 68 water samples of different sampling dates and sites carrying the fitted 9 environmental factors (temperature, DO, ORP, SS, COD, T-N, T-P, and chlorophyll-a in the water column, and COD in the sediment) over the bioaugmentation period was performed as previously described by Chon *et al.* (2013). Non-metric multidimensional scaling (NMDS) is an indirect gradient analysis method which creates an ordination based on a distance or dissimilarity matrix. The scaling method is quite useful in analysis of the complex ecological data into a simplified ordination form.

Microbial community analysis of the sediments before and after bioaugmentation

Bacterial community compositions in the different sites were examined using 16S rRNA gene-based pyrosequencing as shown in the previous work (Kim *et al.*, 2014). In brief, total DNA extraction of sediments was performed following the protocol (Ekpeghere *et al.*, 2009). The variable regions (V1~V3) of the bacterial 16S rRNA gene were then amplified from genomic DNA of each sample from the full-scale system, using fusion primers. Amplification conditions, construction of the sequencing library, sequencing, and analyses were performed using 454 GS FLX Junior Sequencing System (Roche). Analyses of alpha diversity (Shannon index and Chao1 index) (Shannon, 1948; Chao, 1984) and beta diversity (fast unifracs analysis; Hamady

et al., 2010) were conducted using CLcommunity software (ChunLab, Inc.). In ecology, the alpha diversity is usually used to analyze the mean species diversity in sites or habitats at a local scale while beta diversity allows the analysis of the differentiation among those habitats.

Results and Discussion

Monitoring of physical parameters

Some important physical parameters such as pH, DO, ORP, salinity, and SS were monitored along with chemical parameters. pH of all the test sites in the streams were monitored before and after bioaugmentation of BM-S-1. pH of all the sites at the time right before the bioaugmentation (Sept. 28, 2012) was maintained at the range of 7.5~8.2 in the fall and then gradually decreased to 7.6~7.7 in the winter time and to 7.3~7.4 in the spring of year 2013, and increased a little up to 8.0~7.8 in the fall season of 2013. It appeared that the pH was converging to neutrality as the remediation advanced. DO's of all the six test sites before bioaugmentation were ranging from 4.2~5.8 mg/L where the lowest DO was observed at the one of the polluted sites and the highest at the St. 1 (Fig. 2). However, the DO was gradually decreasing up to 2.0~4.5 mg/L for about 3 months (until Nov. 23, 2012). This might be because aerobic microbes utilized DO through degradation of organic pollutants available. This decrease of DO turned out to be linked to a concomitant decrease of

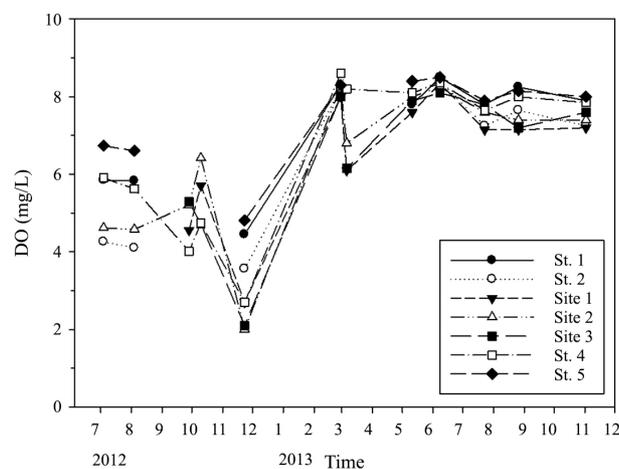


Fig. 2. Effect of bioaugmentation of the microbial consortium BM-S-1 on DO changes of the sea stream water column.

COD of water column and sediment. DO, however, increased up to 8.4~10.1 mg/L which was almost twice as high as DO before bioaugmentation, indicating a recovery of the aquatic ecosystem. This was reflected by the DO profiles at the polluted sites and the less polluted sites before and after the bioaugmentation. There seemed to be much less DO differences among these sites after the BM-S-1 treatment than before. Oxidoreductional potential (ORP) reached -154~-85 mV three weeks after bioaugmentation of BM-S-1 except Site 2, indicating a potential generation of reducing materials from organic pollutants which were degraded by the BM-S-1 consortium. The ORP, however, bounced back to 166~194 mV after about 4 weeks except Station 4, indicating a potential depletion of the organic pollutant after degradation. There was not much difference in the overall ORP among the monitoring sites 17 weeks after the bioaugmentation and it appeared to change proportionally according to DO changes.

Monitoring of water quality parameters

Average removal efficiency of COD in water samples at the five sites (St. 2, Site 1, Site 2, Site 3, and St. 4) was 50% while the highest (58%) was from Site 2 and the lowest (40%) from St. 2 over the augmentation period (Sept. 28, 2012 ~ Nov. 02, 2013; ca. 13 months). Interestingly, the water quality improved from Level III to Level II (Ministry of Environment, 2017) after the augmentation in terms of COD and DO levels. COD removal effect about one month after the augmentation was the

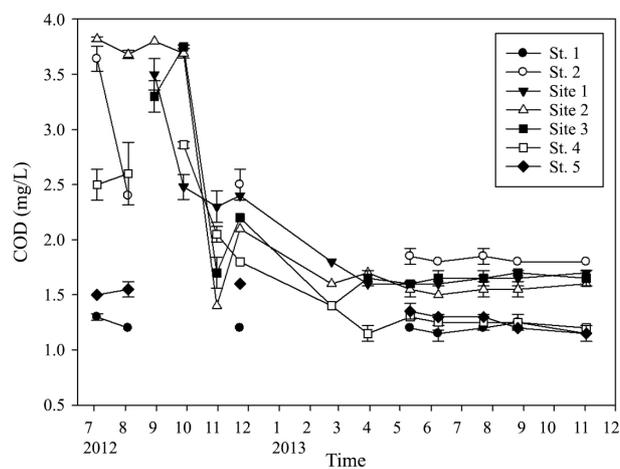


Fig. 3. Effect of bioaugmentation of the microbial consortium BM-S-1 on COD changes of the sea stream water column.

highest at Site 2 (63%) and the lowest at St. 4 (20%). The COD concentrations of these sites, however, increased when the bioaugmentation was temporarily stopped during the 5th and 6th weeks of the BM-S-1 treatment. This might be mostly due to the continuous introduction of wastewater and lower water temperature (~16°C). By the way, the COD removal efficiencies were almost recovered to the previous levels after the bioaugmentation was resumed thereafter. A time course of COD changes also seemed to be linked to those of pH, DO, and ORP. Removal patterns of suspended solids (SS) were quite similar to those of COD (data not shown).

Average removal efficiency of T-N in water samples at the four sites (St. 2, Site 1, Site 2, and Site 3) was 30% while the highest (49%) was from Site 3 and the lowest (1%) from St. 2 over the 13 months augmentation period. T-N removal effect about one month after the augmentation was the highest at Site 1 (53%) and the lowest at St. 4 (-21%). There was little similarity between the removal patterns of T-N and those of COD. This trend was, however, disturbed by the temporary stopping of the bioaugmentation during the 5th and 6th weeks of the BM-S-1 treatment and recovered after the resumed augmentation. The relatively clean sites (Stations 1, 2, and 5) maintained constant lower T-N levels throughout the experimental period (Fig. 4).

Mass balance of nitrogen after reaching a steady state condition of bioaugmentation in 13 months was performed by measuring concentrations of T-N, $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ (data not shown).

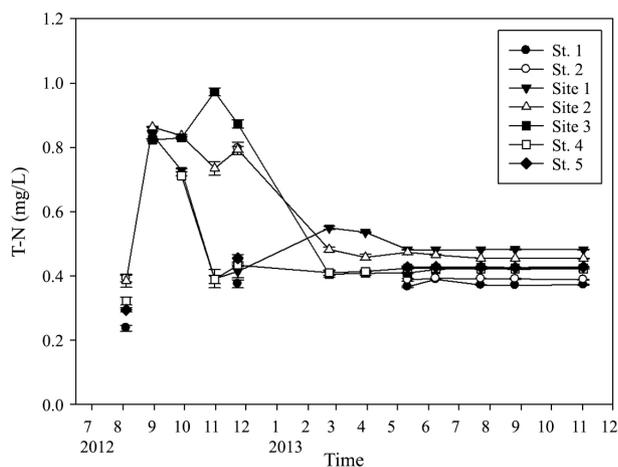


Fig. 4. Effect of bioaugmentation of the microbial consortium BM-S-1 on T-N changes of the sea stream water column.

It was assumed that the ammonium ion could be nitrified to nitrite, nitrite to nitrate which could be eventually removed in forms of NO , N_2O , and N_2 through denitrification process. It is also most likely that the remaining portion of T-N other than $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ would be organic nitrogen and particulate nitrogen while NO , N_2O , and N_2 could not be detected in the total nitrogen analysis. It can be assumed that the absolute nitrogen removal from the aquatic environments could happen through denitrification or anammox reaction of the inorganic nitrogens. In our study, this potential nitrogen removal efficiency was relatively higher in Site 1, Site 2, and Site 3 and while the efficiency was lower in cleaner sites with less concentration of COD as electron donor (Station 1, Station 4, and Station 5). The highest potential efficiency (18%) was observed in the Site 3 that was highly organically polluted, indicating potential action of denitrifiers which used electron donors (e.g., organic acids, amino acids, etc.) derived from degradation of organic pollutants in the stream. The potential nitrogen removal efficiencies (3~18%) in the sites (St. 2, Site 1, Site 2, Site 3, and St. 4) in our study were much lower than these reported rates probably because of lower levels of COD (1.5~2.0 mg/L) and T-N (0.4~0.6 mg/L) compared with COD (30~60 mg/L) and T-N (3.0~5.6 mg/L) (Tang *et al.*, 2013). Denitrification rates in streams may be influenced by many environmental factors including dissolved oxygen (DO), DOC, SRP, and NO_3^- levels (Wallenstein *et al.*, 2006). A sequencing batch biofilm reactor (SBBR) successfully allowed a simultaneous nitrification, denitrification and organic matter removal in the treatment of saline mustard tuber wastewater where average COD removal efficiency of 86.5% and total nitrogen removal efficiency of 86.5% were attained (Wang *et al.*, 2017). Production of greenhouse gases (N_2O , CH_4 , and CO_2) can be proportionally increased by organic matter and NO_3^- levels, in which a highly significant relationship between denitrifying enzymatic activity (and denitrification potential measured as N_2O production) and total organic carbon (TOC) content was observed (Tortosa *et al.*, 2011). Ditch-wetland-pond system (DWPS) was employed to treat polluted river water *in situ* where the average removal rates of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and T-N were 52.0%, 33.3%, and 38.7%, respectively (Tang *et al.*, 2013) which were higher than the rates reported in other studies (Saeed and Sun, 2011).

In contrast to COD and T-N, T-P increased as the augmentation

continued. Average accumulation efficiency of T-P in water samples at the four sites (St. 2, Site 1, Site 2, and Site 3) was 64% while the highest (105%) was from Site 1 and the lowest (-10%) from St. 2 over the 13 months augmentation period (Fig. 5). The relatively clean sites (Station 1, Station 2, and Station 5) showed little difference in the concentration before and after augmentation, maintaining the lowest level of T-P.

Mass balance of T-P after reaching a steady state condition of bioaugmentation in 13 months was also performed by measuring concentrations of T-P and *ortho*-P (PO_4^{3-}) (Fig. 6). Higher concentrations of T-P and PO_4^{3-} were observed in the polluted sites (Site 1, Site 2, Site 3, and Station 4) while lower ones were shown in the clean sites (Station 1, Station 2, and Station 5). The *ortho*-P could be generated from degradation of

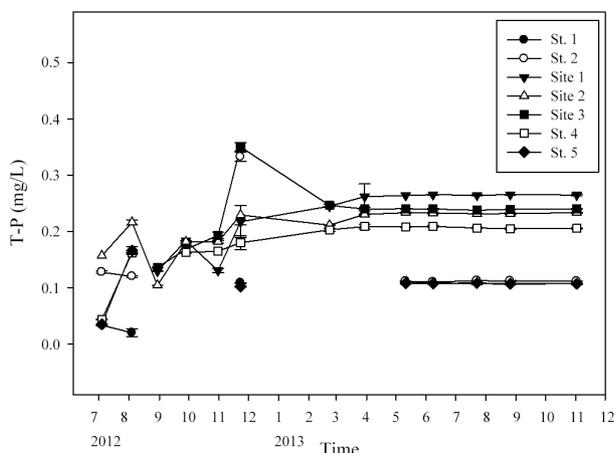


Fig. 5. Effect of bioaugmentation of the microbial consortium BM-S-1 on T-P changes of the sea stream water column.

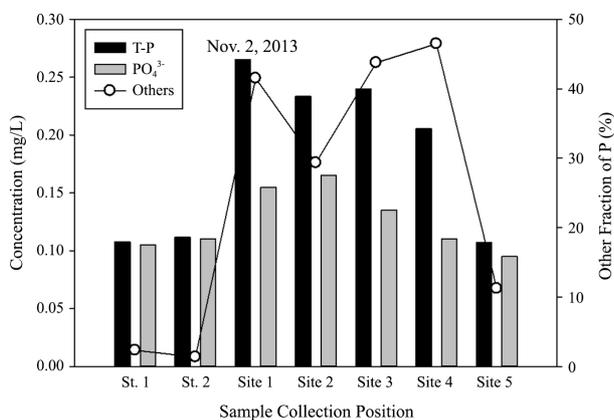


Fig. 6. Effect of bioaugmentation of BM-S-1 on the major phosphorus species dynamics of the sea stream water column about 13 months after the bioremediation.

P-carrying organic materials in the wastewater discharged in the sea stream, and could form a complex precipitated salts such as the insoluble hydroxyapatite [$\text{Ca}_5(\text{PO}_4)_3\text{OH}$], tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$], CaHPO_4 or struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) that can contribute to removal of the *ortho*-P (Liu *et al.*, 2013). Seawater was reported to contain Mg^{2+} (1,248 mg/L) and Ca^{2+} (410 mg/L) but with little PO_4^{3-} -P (Liu *et al.*, 2013). The other fraction of P could represent a potential amount of the insoluble salts whose higher fraction values (more than 25%) were observed in the more polluted sites (Site 1, Site 2, Site 3, and Station 4) while the cleaner sites (Station 1 and Station 2) showed less than 5%. This may indicate that lower concentration (0.1 mg/L) of *ortho*-P cannot readily form the complex salts in the cleaner sites. Recently both seawater and brine possessing a good amount of magnesium were used to recover phosphorus from hydrolyzed urine through formation of struvite (Liu *et al.*, 2013; Tian *et al.*, 2016; Yao *et al.*, 2017).

Average removal efficiency of sediment COD at the four sites (Site 1, Site 2, Site 3, and St. 4) was 32% while the highest (53%) was from St. 4 and the lowest (-107%) from St. 2 over the 13 months augmentation period (Fig. 7). The sediment COD removal effect in comparison to the non-treatment period (July 4 ~ Aug. 30, 2012) was quite obvious in Site 2 (28%), Site 3 (36%), and Station 4 (32%) in one month. In contrast to the water quality parameters (COD, T-N, T-P, and SS), sediment COD removal was not relatively significantly affected by the temporary stopping of bioaugmentation. The lower removal

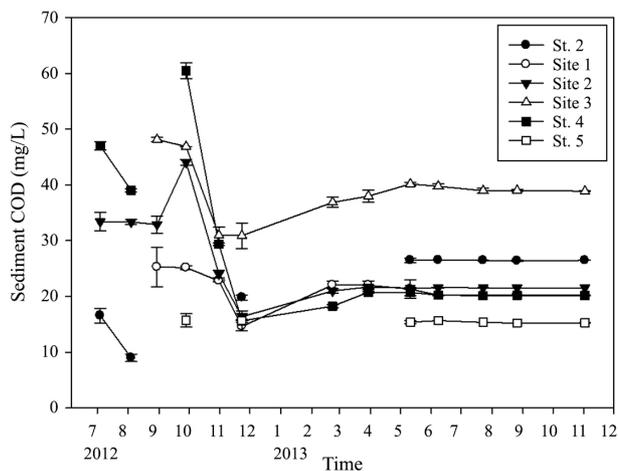


Fig. 7. Effect of bioaugmentation of the microbial consortium BM-S-1 on sediment COD changes of the sea stream water column.



Fig. 8. View of the most polluted site (Site 2) and the least polluted site (St. 4) 85 days before (top) and 33 days after (down) the bioaugmentation of the microbial consortium BM-S-1.

efficiencies in St. 2 (-107%), Site 1 (20%), and Site 3 (19%) were probably due to a direct introduction of a larger amount of wastewater.

These overall consistent removal effects in the various parameters throughout the 13 months experimental period might indicate an active role of the BM-S-1 consortium in bioremediation of the contaminated sediment. The views of the most polluted site (Site 2) and the least polluted site (St. 4) were shown 85 days before and 33 days after the bioaugmentation of the microbial consortium BM-S-1 (Fig. 8). The water turbidities of the two sites were quite high before the bioaugmentation while they significantly decreased after the bioremediation event for about a month. The fish population density rapidly increased after one month of remediation while species diversity and population density of microalgae, and number of species and population density of macroalgae also gradually increased 7~8 months after the remediation (data not shown).

NMDS ordination of samples based on environmental factors

The NMDS plot in this study clearly indicated an ecological difference among the sampling sites (Fig. 9). The samples from St. 4 as a relatively clean site were distinctively clustered

together, while these samples had little correlation with the most of the environmental factors. However, samples from more polluted sites (Site 1, Site 2, and Site 3) were generally clustered together and were more characterized by higher T-P, ORP, and DO. Site 1 also appeared to be characterized by COD and T-N in water to a certain extent.

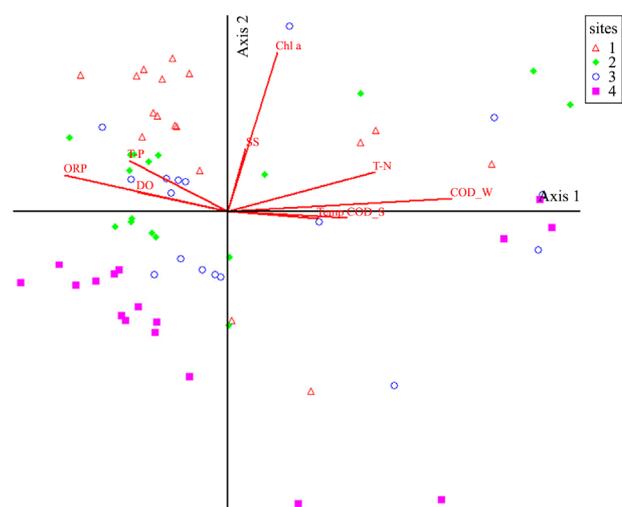


Fig. 9. Non-metric multidimensional scaling (NMDS) plot showing 68 water samples including the fitted 9 environmental factors over the bioaugmentation period. Site numbers: 1, samples from Site 1; 2, samples from Site 2; 3, samples from Site 3; 4, samples from St. 4.

Monitoring of microbial communities in terms of population dynamics and alpha diversity

The valid read numbers of DNA sequences of all samples subjected to the pyrosequencing analysis ranged from 1,426 to 9,422 which were reasonable levels for the microbial community

analysis. Comparative analysis of microbial community changes was accomplished before and after bioaugmentation based on pyrosequencing techniques over the period of 11 months covering the four seasons. The more polluted sites in terms of COD were St. 2, Site 1, Site 2, and Site 3 while less polluted ones were St. 1,

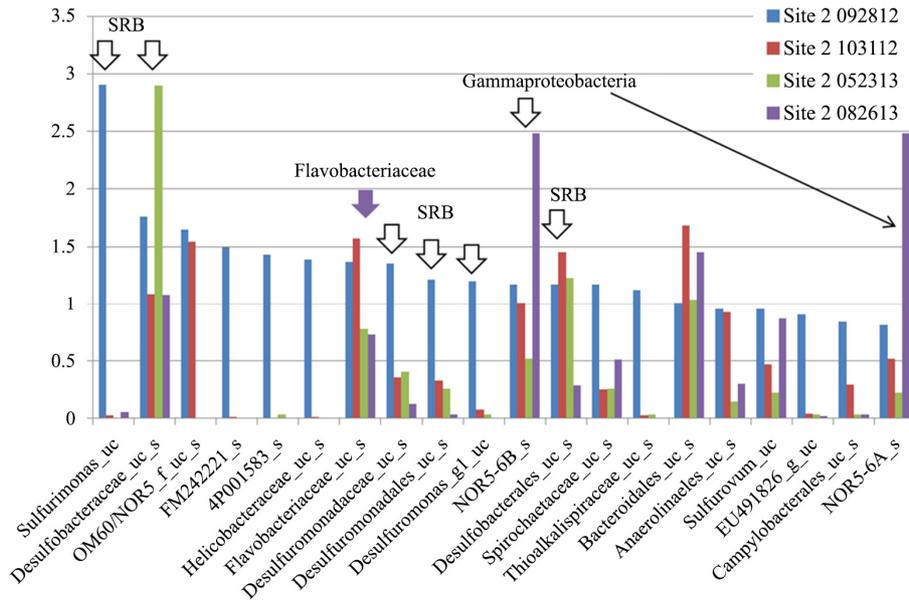


Fig. 10. Microbial community changes in the Site 2 of the sea stream before and after of bioaugmentation of BM-S-1.

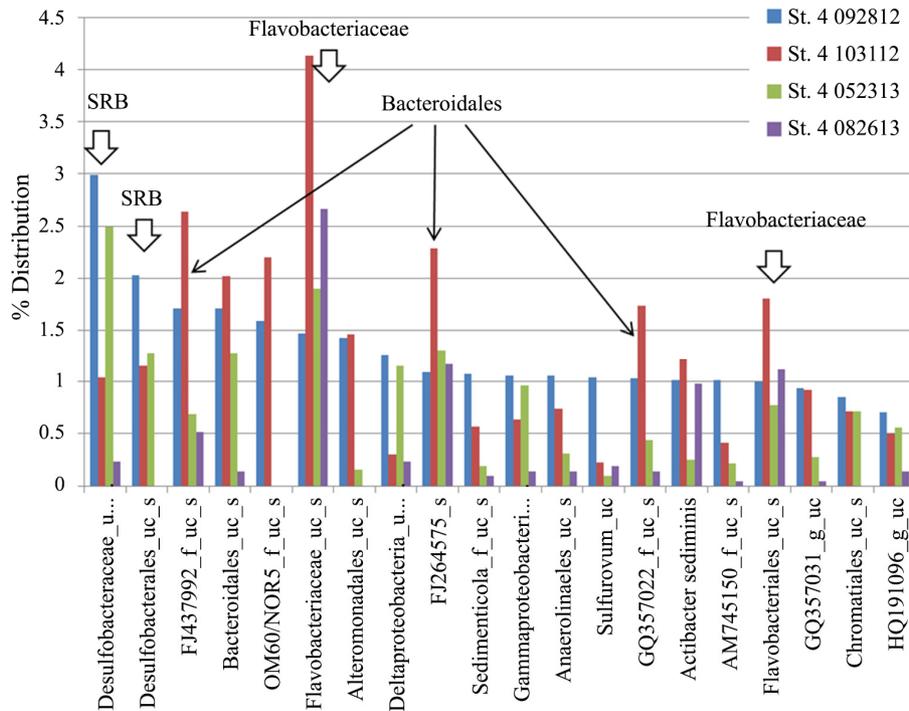


Fig. 11. Microbial community changes in the Station 4 of the sea stream before and after of bioaugmentation of BM-S-1.

St. 4, and St. 5 (Fig. 3). Microbial community was monitored for the St. 2, Site 1, Site 2, Site 3, and St. 4 (data partially shown in Figs. 10 and 11). In all the test sites overall, population densities of sulfate reducing bacterial (SRB) groups (Desulfobacteraceae_uc_s, Desulfobacterales_uc_s, Desulfuromonadaceae_uc_s, Desulfuromonas_g1_uc, and *Desulfobacter postgatei*) and Anaerolineales_uc_s seemed to generally decrease after the bioaugmentation while those of Bacteroidales_uc_s and Flavobacteriales_uc_s appeared to generally increase one month after the augmentation and decrease thereafter. Interestingly, the two species of Gamma-proteobacteria (NOR5-6B_s and NOR5-6A_s) maintained a higher population density in Site 2 and Site 3 (2.5~3.8%) even 11 months after the augmentation. The dominant microbial species in St. 2 before augmentation were Desulfobacteraceae_uc_s (3.1%) and Desulfobacterales_uc_s (2.2%). NOR5-6A_s and NOR5-6B_s (3.0%, each) were dominant 11 months after augmentation. The dominant ones in Site 1 before augmentation were Desulfobacteraceae_uc_s (3.8%), Desulfuromonadaceae_uc_s (2.8%), AJ271656_g_uc (2.8%, Desulfuromonadaceae), and Desulfobacterales_uc_s (2.0%). FJ437992_f_uc_s (Bacteroidales) increased up to 3.4% one month after the augmentation but decreased thereafter. *Sulfurovum* HQ203755_s increased up to 6.7% 11 months after the augmentation. The dominant species in Site 2 as the most polluted site before augmentation was *Sulfurimonas_uc* (2.9%) but then decreased to less than 0.1% after augmentation. NOR5-6A_s and NOR5-6B_s (2.5%, each) were also dominant 11 months after augmentation. The dominant ones in Site 3 before and after augmentation were FJ264575_s (2.6~3.9%, Bacteroidales) and FJ437992_s (1.9~2.7%, Bacteroidales) and showed an increasing trends as the augmentation went. Again, NOR5-6B_s and NOR5-6A_s were significantly dominant 11 months after the augmentation. The dominant ones in St. 4 before augmentation were Desulfobacteraceae_uc_s (3.0%) and Desulfobacterales_uc_s (2.0%) which decreased significantly as the augmentation continued. Maximal densities of Flavobacteriaceae_uc_s (4.1%), FJ437992_f_uc_s (2.6%), FJ264575_s (2.3%), OM60/NOR5_f_uc_s (2.2%), Bacteroidales_uc_s (2.0%), and Flavobacteriales_uc_s (1.8%) were observed after one month augmentation but decreased thereafter. Flavobacteriaceae_uc_s and Flavobacteriales_uc_s appeared to maintain higher population densities 11 months after the augmentation (2.7% and 1.1%, respectively)

while OM60/NOR5_f_uc_s (Gamma-proteobacteria; Alteromonadales) disappeared about 8 months after the augmentation. NOR5-6A_s and NOR5-6B_s (Gamma-proteobacteria; OM60_f), in particular, significantly increased in all the more polluted sites (St. 2, Site 1, Site 2, and Site 3) in 11 months after the bioaugmentation while they were kept in less than 1% in St. 4 during the whole test period. Seawater enriched with nutrients was able to stimulate growth of the copiotrophic bacteria such as groups of Gamma-proteobacteria (i.e. *Alteromonadales*, *Pseudoalteromonas*, *Pseudomonadales*, OM60 group) (Nogales *et al.*, 2011). Therefore, it was assumed that the relative clean site St. 4 did not favor growth of the two species of Gamma-proteobacteria (OM60 family). Moreover, populations of SRB significantly decreased one month after the bioaugmentation, indicating that removal of organic pollutants (hence decrease of COD) could enhance DO and ORP levels so that SRB could not survive in the environment transformed from anoxic to oxic conditions. This phenomenon was relatively more obvious in more polluted sites (Site 2 and Site 3) than less polluted site (St. 4) (Figs. 10 and 11). Sulfate-reducing prokaryotes (SRP) play an important role in the degradation of organic matter in coast and also in deeply buried marine sediments in the open ocean (Blazejak and Schippers, 2011), and degradation of organic matter as well as cycling of sulfur and carbon substrates in the fresh water lake (Čanković *et al.*, 2017). Aerobic microbial communities (Flavobacteriaceae_uc_s) were dominant in St. 4 that showed the highest level of DO and least level of COD. These microbial communities could be an indicator organism in the restored environment. Most members of the family can easily utilize organic macromolecules such as polysaccharides and proteins (Tamaki *et al.*, 2003; McBride, 2014). Gamma-proteobacteria quite increased in the more polluted sites (St. 2, Site 1, Site 2, and Site 3) after the bioremediation event. Organisms of this class frequently were found in the intertidal and marine sediments (Wang *et al.*, 2012) and they harbor the family Chromatiaceae which carries purple sulfur bacteria generally inhabiting in illuminated anoxic zones of fresh water and salt water aquatic habitats where hydrogen sulfide can be produced by SRB. It is likely that the sulfur bacteria can utilize the hydrogen sulfide produced by the SRB observed in our pyrosequencing data. Uncultured *Gammaproteobacteria* are important sulfur-oxidizing prokaryotes in the tidal sediments that

were involved in sulfide removal and primary production (CO₂ fixation) in marine surface sediments (Lenk *et al.*, 2011). Therefore, it has been assumed in our study that the SRB and the Gamma-proteobacteria could live in a symbiotic relationship.

The alpha diversity indices such as OTUs, Chao1, and Shannon for the microbial communities were monitored before and after the bioaugmentation. The three indices ranged: 786~4730 (2476±789), 2016~12450 (6334±1930), and 6.26~7.95 (7.1±0.4), in order. Most of the indices decreased over the 11 months period except St. 2 whose OTUs, Chao1, and Shannon indices increased by 18%, 6%, and -4%, respectively. The indices of OTUs and Chao1 for St. 4, Site 1, and Site 2 most decreased in order by 44~77% while Shannon index decreased in order by 12~19% in the same sites. This means that changes of microbial communities in St. 4, Site 1, and Site 2 might be more sensitive to the augmentation than in St. 2 and Site 3. The reason for this is not clear but it is assumed that the pollution loading level and

biodegradation rate of pollutants in the sites could affect the sensitivity. By bioremediation strategies including bioaugmentation and biostimulation, the Fisher's [alpha] diversity index increased in soil contaminated with diesel oil significantly after 28 days (Luisa *et al.*, 2015).

Analysis of microbial communities before and after bioaugmentation in terms of beta diversity

There were seven clusters of the microbial communities formed for all the samples classified in terms of sampling sites and dates before and after the bioaugmentation (Fig. 12): Cluster 1 (Site 1 and Site 2 before the augmentation), Cluster 2 (Site 3 and St. 4 before the augmentation; Site 1, Site 2, Site 3, and St. 4 about one month after the augmentation), Cluster 3 (St. 2 before the augmentation),

Cluster 4 (Site 1, Site 2, Site 3, and St. 4 eight months after the augmentation), Cluster 5 (St. 2 one and eight months after

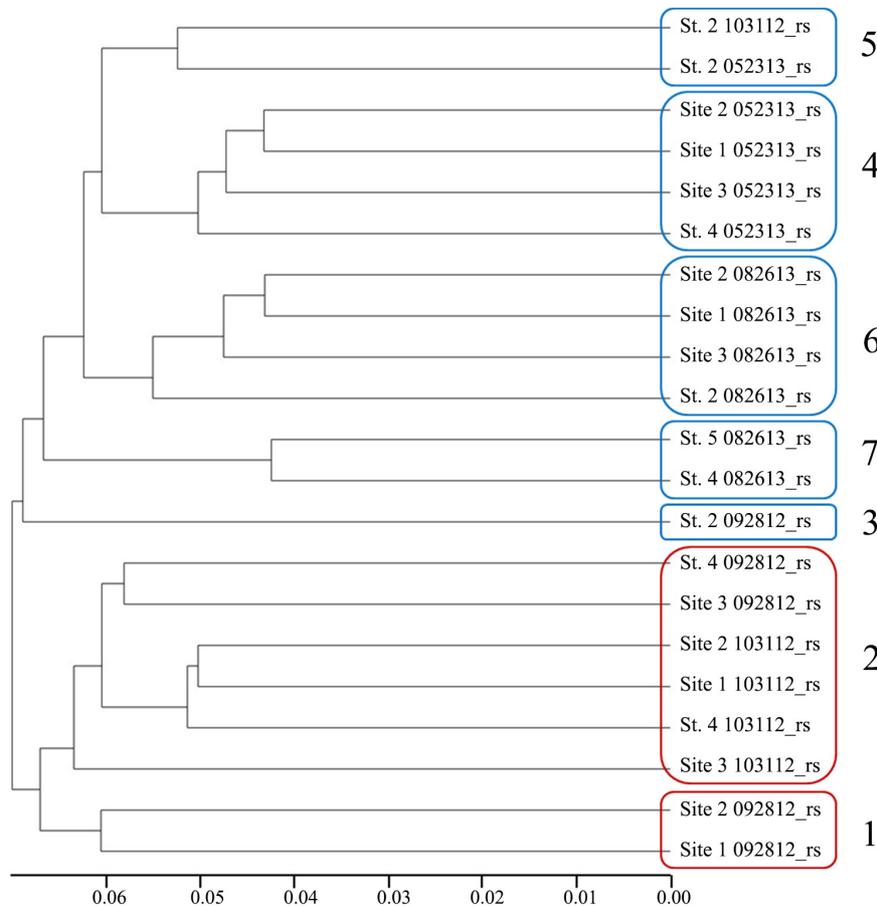


Fig. 12. UniFrac cluster analysis of the microbial community structures at different spatial and temporal scales before and after bioaugmentation of BM-S-1.

the augmentation), Cluster 6 (Site 1, Site 2, Site 3, and St. 2 eleven months after the augmentation), and Cluster 7 (St. 4 and St. 5 eight months after the augmentation). These clusters were overall belonged the two groups; one group carried Cluster 1 and Cluster 2 whose samples were taken before and one after the augmentation (highlighted in red circle, Fig. 12), and the other group whose samples were mostly from taken several months after the augmentation (highlighted in blue circle, Fig. 12). This indicates that the microbial augmentation could change the indigenous microbial community structures in the direction of a homogeneous community structure as the augmentation continues. That is, samples from St. 2, Site 1, Site 2, and Site 3 will belong to one big cluster since these sites are relatively close by and are influenced by the augmentation effect from Site 2. However, the St. 4 and 5 would be less affected by the augmentation because they are rather remotely located from the Site 2 and their water quality was relatively higher than the other sites. All these facts indicate that the bioaugmentation can improve the polluted water quality and simultaneously change the microbial community structures via their niche changes.

적 요

부산시 영도구의 혁신지구의 인공해수전은 높아진 하상과 조류의 특성으로 인해 물이 순환되지 않고 더구나 주위의 오수가 유입되고 있어서 수질이 나빠지고 악취를 발생하고 있다. 이 문제를 해결하기 위한 방안으로 가장 오염되고 조류이동을 감안한 하천의 지점에 생물증강법을 적용하여 친환경적, 효율적으로 하천을 정화하고자 하였다. 현장에서 활성화된 복합미생물을 가장 오염된 지점(Site 2)에 7~10일 간격으로 투입하여, 수질의 pH, 온도, DO, ORP, SS, COD, T-N, 및 T-P를 측정하였고 또한 하상퇴적토의 COD 및 미생물군집을 분석하였다. 13개월 후 Site 2의 수질 SS, COD, T-N 및 COD (퇴적토)의 처리 효율은 각각 51%, 58%, 27% 및 35%으로 나타났으나 T-P는 오히려 47% 증가를 보였다. 대부분의 측정지점에서 황산염환원세균(sulfate reducing bacteria)그룹(Desulfobacteraceae_uc_s, Desulfobacteriales_uc_s, Desulfuromonadaceae_uc_s, Desulfuromonas_g1_uc and *Desulfobacter postgatei*)과 Anaerolineales의 밀도는 대체적으로 생물증강에 의한 정화가 진행될수록 감소하였으며, 반면에 Gamma-proteobacteria (NOR5-6B_s and NOR5-6A_s), Bacteroidales_uc_s, 및 Flavobacteriales_uc_s의

밀도는 증가하는 경향이였다. 상대적으로 COD가 낮고 DO가 높은 청정지점인 St. 4에서는 호기성미생물인 Flavobacteriaceae_uc_s가 우점하였다. 이러한 미생물군은 하천의 정화과정을 추적할 수 있는 지표미생물로 활용될 수 있을 것으로 판단되었다. 생물증강 시행 후의 대표적 시점 퇴적토시료의 미생물군집 alpha diversity 지수(OTUs, Chao1 및 Shannon 지수)는 시행 전에 비해 감소하는 경향을 보였으며, 또한 beta diversity 분석기법(fast unifrac 분석)으로 분석한 결과 정화 전이나 초기에 비해서 정화가 진행될수록 전반적으로 시료에 무관하게 미생물군집의 유사성을 보여 생물증강이 현장 토착 미생물의 군집구조를 변화시키고 있음을 확인하였다. 이러한 사실로 보아 본 복합미생물에 의한 현장 생물증강법은 brine water 및 담수로 이루어진 오염된 하천을 환경친화적, 경제적으로 정화할 수 있는 대안으로 판단이 되었다.

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