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In-situ microbial colonization and its potential contribution on biofilm formation in subsurface sediments

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Abstract Biofilms facilitate communication among microorganisms for nutrients and protect them from predators and harmful chemicals such as antibiotics and detergents. Biofilms can also act as cores for the development of clogs in many agricultural irrigation systems and in porous media. In this study, we deployed glass units at a depth of 20 m below the ground surface in the groundwater-surface water mixing zone, and retrieved them after 4 months to investigate the potential colonization of indigenous microbial community and possible mineral-microbe assemblages. We observed the periodic formation of microbial colonies by fluorescence dye staining and microscopy, and analyzed the composition of the microbial community in both the mineral-microbe aggregates and groundwater, by next generation sequencing of the 16S rRNA gene amplicons using MiSeq platform. During the course of incubation, we observed an increase in both the mineral-microbe aggregates and content of extracellular polymeric substances. Interestingly, the microbial community from the aggregates featured a high abundance of iron redox-related microorganisms such as *Geobacter* sp., *Comamonadaceae* sp., and *Burkholderiales incertae sedis*. Therefore, these microorganisms can potentially produce iron-minerals within the sediment-microbe-associated aggregates, and induce biofilm formation within the groundwater borehole and porous media.

Keywords Bioclogging · Groundwater · Microbial community · Subsurface biogeochemistry

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Introduction

Biofilms play an important role in the formation of bio-clogging in various irrigation systems [1,2] and in porous media [3,4]. Biofilms formed due to biomass accumulation and microbially induced precipitation of minerals in saturated subsurface environments can give rise to bio-clogging in the porous media [1,5], and result in a decrease in the permeability of the subsurface environment. During growth, microorganisms excrete extracellular polymeric substances (EPS), which increase the cohesiveness of the cells and/or biomass, leading to the formation of biofilms [1,6]. EPS have slimy and gel-like characteristics, and their main constituents are polysaccharides, proteins, and nucleic acids. Thus, EPS can potentially accumulate biomass and inorganic substances, such as minerals and sediments and form biofilms.

Biofilms have been in focus for their application as bio-barriers to prevent the spread of pollutants and degrade them within the containments in the subsurface and groundwater [3,7,8]. However, biofilms have also resulted in the decreased efficiency in engineered bioremediation of contaminated groundwater [9]. Nie et al. [10] have reported on the changes in the microbial community of the soil supplemented with nutrients from septic tank effluent lead to the biofilm formation, using denaturing gradient gel electrophoresis analysis. However, the microbial communities contributing to the formation of biofilms in porous media and/or groundwater have not been well characterized.

In the present study, we investigated the microbial community that colonized on the *in-situ*-formed mineral-microbe aggregates, in the groundwater borehole. The study area or borehole was in a

hyporheic zone, where groundwater and surface water mixing occurred. Therefore, we investigated the effect of dissolved oxygen (DO) and dissolved organic carbons (DOCs), potentially migrating along with the surface water into the groundwater, on microbial colonization and potential for biofilm formation in the porous media. This study could provide insight into the microbial community composition contributing to the formation of biofilms and/or mineral-microbe aggregates in subsurface-saturated porous media environments.

Materials and Methods

In-situ incubation and colonization in groundwater

Microscopic cover glasses (Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany) were deployed into the groundwater borehole to examine possible microbial colonization on them. The groundwater borehole was described in the previous publication [11], and it was located within the mixing zone between stream water and groundwater in a heavy groundwater extraction area for agricultural use. Each cover glass was inserted into a plastic container (cell) with meshes on both sides of the container for water flow-through and 20-mesh cells were stacked up on a cylinder-type holder (Fig. 1). The cylinder-type holder was deployed into the borehole WJ2 [11] at a depth of 20 m below the ground surface, and retrieved for sampling the plastic cells containing the cover glasses at selected time points. Some of the groundwater and geochemical parameters were measured on-site and in the laboratory after being transported at 4 °C, as indicated in Table 1.

Sampling of the colonized slides and confocal laser scanning microscopy (CLSM) analysis

The retrieved cover glasses were directly examined for potential microbial colonization on the glasses using the CLSM (TCS SP5/AOBS/tandem scanning system, Leica Microsystems GmbH, Wetzlar, Germany). The sampled cover glass surface was stained with SYTO9 (fluorescent green; Life Technologies, Carlsbad, CA, USA) and lectin PHA-L (Alexa Fluor 594 conjugate; Molecular Probes, Inc., Eugene, OR, USA) for bacterial cells (nucleic acids) and EPS, respectively. The EPS play a critical role in the formation of structured multicellular bacterial communities or biofilms [1]. Thus, CLSM examination was performed to observe the association of bacterial cells with EPS on the aggregates formed on the glass surfaces.

Genomic DNA extraction and microbial community analysis

Surfaces of the retrieved cover glasses were swiped with sterile cotton and the groundwater was filtered using a 0.45-μm pore-size, 47-mm diameter cellulose acetate membrane filters (Advantec, Tokyo, Japan) to collect the bacterial cells for DNA extraction. Cottons and filters were used for DNA extraction using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Three DNA extracts were pooled into one for both the glass-colonized and planktonic microorganisms, and were then used for further downstream experiments. The V1-V3 region of the 16S rRNA gene was amplified from the genomic DNA using the 16S rDNA fusion primer set (27F, 5-GAG TTT GAT CMT GGC TCA G-3 and 518R, 5-WTT ACC GCG GCT GCT GG-3), followed by sequencing using a GS-FLX sequencing system (Roche, Basel, Switzerland) by Macrogen (Seoul, South Korea).

The sequence was processed using Mothur ver. 1.33.3 [12], by referring the suggested standard operating procedure for 454 pyrosequencing [13-15]. Sequence errors were reduced by using a series of subroutine processes in Mothur, and were screened for chimeras using UCHIME [16]. After the removal of low-quality reads and non-bacterial sequences, the total number of sequences from the two samples was 13,361. Total sequence counts were 5,921 and 7,440 for the glass surface (WJ2-S) and groundwater (WJ2-W), respectively. The sequences were aligned against the

Table 1 Groundwater and geochemical parameters monitored at the depth of 20 m below ground surface during the deployment period

Incubation time	Date	DTW[a] (m)	Temp (°C)	EC[b] (μS/cm)	Eh (mV)	pH	DO (mg/L)	S(-II) (μM)	Fe(II) (μM)
- 97 d	2014-Jan-2	5.1	14.4	224	-159	6.14	1	n.a.[c]	n.a.
time 0	2014-Apr-9	4.7	15.5	207	165	6.21	6.89	n.a.	n.a.
33 d	2014-May-12	4.2	17.3	223	78	6.08	7.24	0.5	98
69 d	2014-Jun-17	3.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
104 d	2014-Jul-22	4.0	14.4	211	55	5.84	2.46	1.3	290

[a]Depth to water; [b]Electrical conductivity; [c]Not available

Table 2 A sequence summary and alpha diversity: richness and diversity indices of the samples at selected times

Sample	Subsampled sequences	Good's coverage	Observed OTUs	Richness		Diversity	
				Chao1	ACE	Shannon	Inverse simpson
WJ2S	5,921	0.880	1,264	2,507	3,633	5.35	26.4
WJ2W	5,921[a]	0.778±0.003	2,147.8±13.9	4,673±142	6,722±235	6.85±0.01	299.9±6.1

[a]Subsampled to 5,921 to be compared at the same number

SILVA database ver. 102 [17], and classified using the Ribosomal Database Project 16S rRNA gene training set ver. 9, to be assigned to OTUs at a 3% dissimilarity level. For analyzing the alpha diversity of the samples, the sequence numbers were normalized to the smaller number (Table 2). Raw reads of the DNA sequencing project were deposited in NCBI's Sequence Read Archive database under the accession number PRJNA493783.

Results and Discussion

Mixing of stream water and groundwater

Annual heavy extraction of groundwater was used as an extra heat source for the greenhouses at the study site during the cold season, from early November to the April [11]. Unlike the average low levels of surface water during the dry cold season in the Korean peninsula, the stream level adjacent to the study site was raised possibly by the used groundwater runoff during heavy groundwater extraction [11], whereas heavy pumping reduced the groundwater level. It appeared that the adjacent stream water intruded the aquifer by the extraction-derived force, but there was a delay of the groundwater migration into the aquifer, probably due to properties of the porous materials with silty sediments [11]. The level of groundwater of the study borehole (WJ2) showed a gradual increase during the experiment from April to July (Table 1), which was consistent with the previously published data from a different well [11]. This helped to explain the above-described delayed response of the groundwater level from the extraction-free wells against the groundwater extraction and the stream water migration almost after the cessation of the heavy pumping in April. Thus, the migration of stream water into the groundwater was evidenced by the rise of the groundwater table during the dry season (April to May). The DO concentrations of the groundwater were quite high at the depth of 20 m in April and May (Table 1), suggesting the possible mixing with surface water. The relatively high concentrations of DO maintained during the migration were possibly influenced by the proximity between the borehole and the stream (approximately 30 m).

In addition to DO as one of the important factors for the microbial growth, DOC is a major factor. It has been suggested that DOCs in the groundwater, which migrate along with surface water, influenced the shifts in the microbial community composition in the groundwater-surface water mixing hyporheic zone [18]. Additionally, DOCs were simulated to be migrated into the groundwater by addition to the laboratory incubations of the subsurface hyporheic zone sediments and groundwater, facilitating the shift in the microbial communities by the activities of ferric iron and sulfate reduction [19]. The shifted communities were comparable with, but different from the initial microbial composition [20]. Therefore, it was speculated that the surface water migration might have brought the DO and DOC into the groundwater and triggered microbial activities, and resulted in colonization with the shifted microbial communities.

Fig. 1 Deployed units of the permeable plastic cells containing the microscopic cover glasses

Colonization of microorganisms in the borehole

The deployed and retrieved water-transmissible units were examined by CLSM to observe the microorganisms and EPS potentially associated with the formed sediment aggregations, using the fluorescent dyes as described above. The green and red fluorescence of the microorganisms and EPS, respectively (stained respectively by SYTO9 and lectin PHA-L), indicated that the sediment/mineral aggregates found on the retrieved glass surface after 33 d were associated with microbes (Fig. 2A). As the deployment duration increased, the detection of EPS and/or extracellular polysaccharides (EP) was more frequent and obvious for the samples after 69 d (Fig. 2B, C) and 104 d (Fig. 2D). In the 69-d samples, red fluorescence appeared as spots, whereas in the 104-d samples, rather broad covers of the red fluorescence were observed on the mineral aggregates. This indicated that the EPS/EP began growth as spots and formed very slimy thin layer of biofilm in the later period as shown in Fig. 2D. Individual cells of

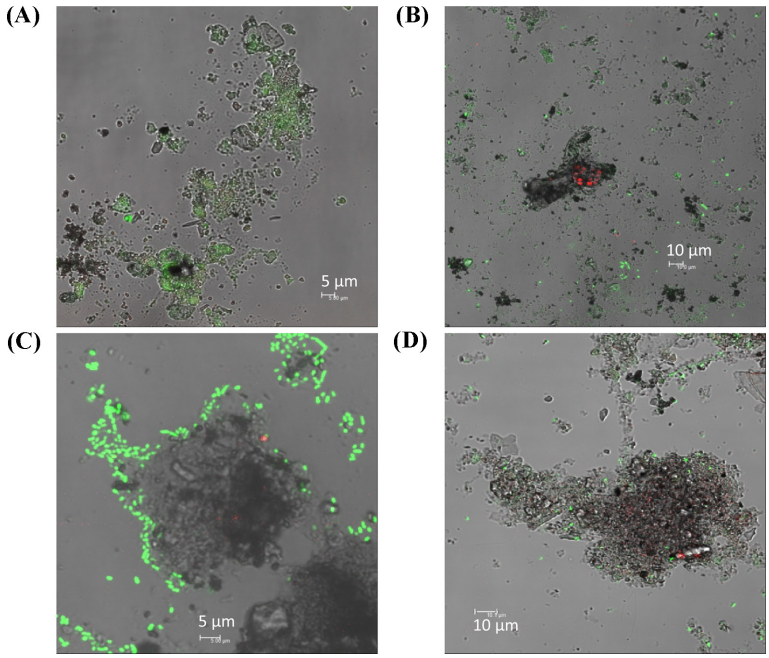

Fig. 2 CLSM images of the mineral-microbe aggregates on the retrieved glasses after 33 d (A), 69 d (B, C), and 104 d (D). Bacterial cells (nucleic acids) indicated by the green fluorescent by SYTO9 and EPS indicated by red fluorescence by lectin PHA-L

microorganisms attached to the mineral aggregates were commonly observed (Fig. 2C), which probably were members of the communities colonized on the sediments and minerals.

In order to identify the microbial community composition of the microbe-colonized mineral aggregates, both the slide glasses (WJ2S) and the groundwater (WJ2W) retrieved after 104 d were subjected to pyrosequencing of the amplicons of 16S rRNA gene (V1-V3 region). The subsampled sequences for normalization to be compared at the same numbers were relatively good for the community resolution, as indicated by the Good's coverages of 88% and 78% (Table 2). The number of operational taxonomic units (OTU) by 97% similarity (species level) was larger in the groundwater-planktonic microbial community than in the glass-colonized community. This was consistent with the indices of species richness or number of different species as Chao1 (estimator of total species richness) and the abundance-based estimator, with larger numbers from the groundwater community (Table 2). The diversity indices of Shannon and inverse Simpson, indicating both species richness and evenness, were also larger in the groundwater-suspended community than from the glass-colonized community (Table 2). Thus, the groundwater-suspended or planktonic microbial community (WJ2W) was larger in species numbers and those different species were relatively evenly distributed in the groundwater (WJ2W) than in the mineral aggregates-associated community (WJ2S).

The OTUs were clustered into 20 phyla and 6 classes within the phylum *Proteobacteria* (Fig. 3A). Among the 20 phyla, *Proteobacteria* was the most abundant in both the samples (61.9 and 43.8% for WJ2S and WJ2W, respectively), and *Betaproteobacteria* (35.0 and 19.3%) and *Deltaproteobacteria* (15.3 and 6.3%) were dominant. For the remaining portions, *Bacteroidetes*, *Acidobacteria*, and unclassified *Bacteria* were also abundant. The community on the glass colonization was slightly different from that in the groundwater, by the increase in *Betaproteobacteria* and decrease in *Bacteroidetes* (12.9 and 21.0% for WJ2S and WJ2W, respectively).

When the OTUs at genus level were selected for those larger than 1% in at least one of the samples, 25 genera were included and clustered by the Bray-Curtis dissimilarity as shown by the horizontal dendrogram (Fig. 3B). There was only minor similarity between the clusters of the glass-colonized (WJ2S) and the groundwater (WJ2W) communities. In the mineral-associated community (WJ2S), however, there was a relatively strong-clustered group of three genera, consisting of unclassified *Comamonadaceae* (16.9%), *Geobacter* (10.6%), and unclassified *Burkholderiales incertae sedis* (5.2%), that were not detected in the groundwater community (Fig. 3B). While the genus *Geobacter* is well known for ferric iron reduction, there are many iron-reducing or -oxidizing bacteria both in the family *Comamonadaceae* and in the genera *Burkholderiales incertae sedis* such as *Acidovorax* [21], *Rhodoferax* [22], *Albidiferax*, *Leptothrix*, unclassified *Burkholderiales* bacterium [23] etc. Considering only the classified taxa down to genus level, the most abundant genus was *Geobacter* followed by *Geothrix* and *Desulforhopalus* (Fig. 3B), and these were iron or sulfur redox-related microorganisms. Thus, we speculated that these microorganisms such as Fe(III)-reducing *Geobacter* and *Geothrix*, and sulfate-reducing *Desulforhopalus* might have important roles for the formation of the microbe-mineral associations by excreting EPS/EP. In addition, it appeared

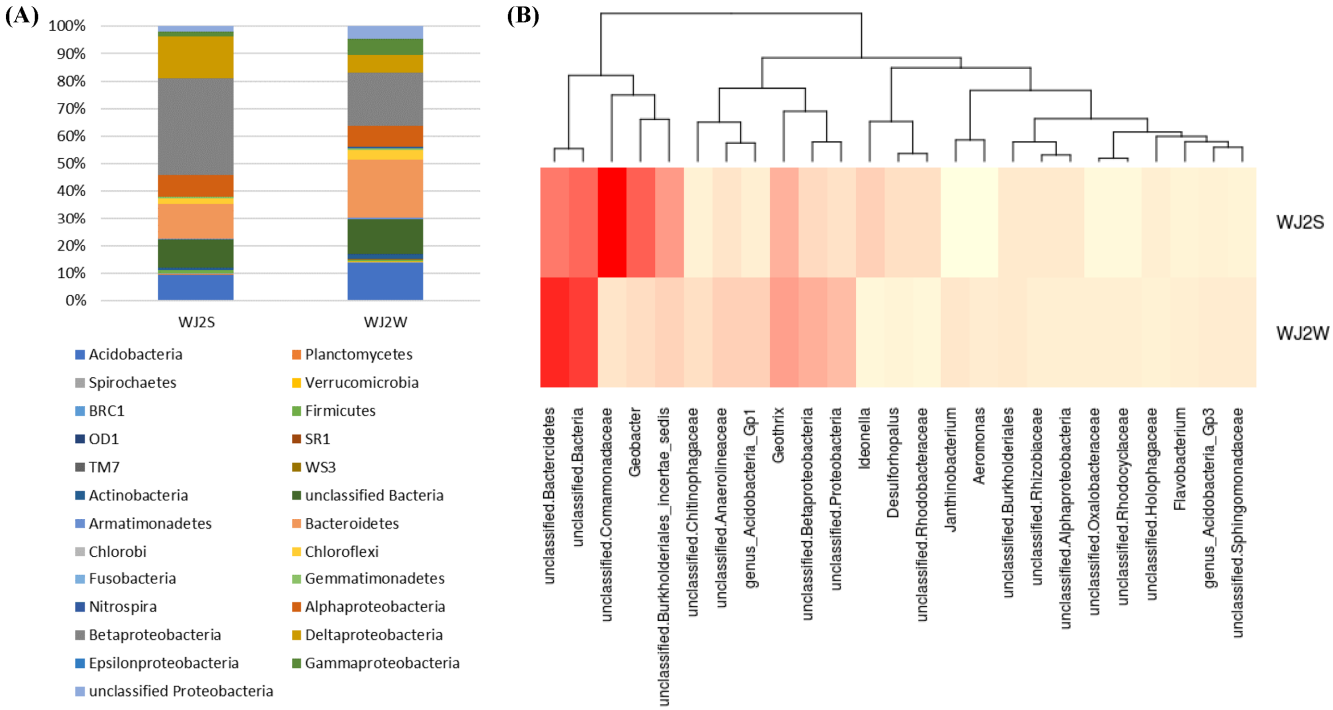

Fig. 3 Next-generation sequencing (MiSeq) of the 16S rRNA gene amplicons. (A) Relative sequence abundances at phylum level and classes within *Proteobacteria*. (B) A heatmap illustrating abundance of the OTUs at the genus level larger than 1% in at least one of the communities. A dendrograms on the horizontal axis is based on the Bray-Curtis dissimilarity matrix. The color gradient indicates fractions summing to 1

that the formation of those mineral-microbe associations may have grown with time, and therefore provided potentially large slimy materials to the borehole, which eventually could trigger the biofilms in the borehole and porous media as well. It was not successful to identify the formed minerals' characteristics by using X-ray diffraction and X-ray photoelectron spectroscopy, because of redox sensitive, amorphous, and small amount Fe-fractions with much larger fractions of sand particles. However, there were dark spotted precipitates in the mineral aggregates and those were considered as reduced phases of amorphous Fe-(oxyhydr)oxides and/or ferrihydrites.

Environmental implications

In this study, microbial colonization was examined in the groundwater of the hyporheic zone, where groundwater and surface water mixing occurred. Presumably, the DO and DOC increased the microbial activities and the sediment/mineral-microbe associations were formed. With time, the aggregates could grow as indicated by the size and EPS observations, which eventually influenced the formation of biofilms within the groundwater borehole. Moreover, the microbial community from the aggregates was featured by a cluster of iron redox-related microorganisms. We propose that these microorganisms could be the members directly producing the iron minerals within the sediment-microbe-associated aggregates. This study shows the chronological observation of the *in-situ* formation of sediment-microorganism-associated aggregates in the subsurface saturated porous media. The implications of this study include the potential roles of microbial compositions possibly responsible for the iron-minerals formation on the biofilms within the sediment-mineral-microbe associations.

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