1 **Comparison of Microbial Communities Inside and Outside of a Denitrification Hotspot**
2 **in Confined Groundwater**

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Abstract A denitrification hotspot was detected in Kumamoto groundwater in a previous study, little information is concerned with the microbial community profile including denitrifier. Seven samples from inside and outside of the hotspot were collected to analyze the bacterial, archaeal, and denitrifier community using a cloning library approach. The results showed that the microbial diversity and distribution were distinct among the zones. Major operational taxonomic units closely related to *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were found in the denitrification hotspot. Additionally, type I methylotrophic bacteria, such as *Methylobacter* and *Methylomonas*, only inhabited the hotspot zone. A positive association between methanogenic archaea and methylotrophic bacteria was observed. Interestingly, acidic ammonia-oxidizing archaea were detected in the neutral groundwater environment. In addition, gene-specific analysis targeting *nirS* indicated that the majority of denitrifiers belonged to the *Proteobacteria* including *Sulfuritalea hydrogenivorans*, *Pseudomonas balearica* and *Sulfuricella denitrificans*. Based on the comparative analysis of microbial communities and physiochemical parameters inside and outside of the denitrification hotspot, the anaerobic environment with organic compounds and nitrate could support the biotic reduction of nitrate in confined groundwater.

Keywords: Groundwater, 16S rDNA, *nirS*, Denitrification hotspot, Microbial community

1. Introduction

Nitrate (NO₃) pollution has become a significant issue in many countries owing to its adverse effects on human health and the environment, such as the eutrophication of surface water (Vitousek et al., 1997; Mason, 2002) and groundwater nitrate contamination (Beeson and Cook, 2004; Rao, 2006; Rivett et al., 2007). The microorganisms inhabiting either natural environments or artificial reactors have developed one or more mechanisms to reduce nitrate to maintain the N-cycle balance. Anaerobic ammonia oxidation, dissimilatory nitrate reduction to ammonia, and denitrification process are ubiquitous in the natural environment and play critical roles in the attenuation of nitrate. Denitrification, the dissimilatory process of reduction of nitrate or nitrite to gaseous products (N₂O or N₂) in the suboxic environment, is generally considered to be the major biological loss pathway for the conversion of fixed nitrogen from terrestrial aquatic ecosystems to the atmosphere (Devol, 2008). The functional genes *nirS/K*, which encode a key enzyme for the reduction of nitrite to nitric oxide during the denitrification process, has been used extensively to analyze the denitrifier community in the environment.

Kumamoto City lies in the center of the Kyushu islands in southwest Japan, and is the largest groundwater utilization region in the country. About one million people in and around the city depend entirely on groundwater as the source of drinking water. Although Kumamoto City has had good performance with respect to groundwater protection in the past few decades, the concentration of NO₃ in the groundwater has been increasing since the 1970s as a result of the widespread application of chemical fertilizers and manure. Although the level of NO₃ is still under the limit for healthy drinking water, the trend suggests a potential threat to the stability of the local environment and human health.

Given the importance of groundwater for local residents and the serious consequences of nitrate pollution, multiple studies performed in the Kumamoto groundwater studying area have examined groundwater, from various perspectives, such as geology (Miyoshi et al., 2009), hydrogeology (Taniguchi et al., 2003; Tanaka et al., 2010), and groundwater age and flow (Shimada, 2012). Those findings provided a basis for the analysis of microbiological

communities and their mechanism of nitrate reduction. For example, Hosono et al. (2013) investigated the origin of nitrate and its attenuation mechanism using isotope labeling. Interestingly, the isotope data revealed a denitrification hotspot in the Kumamoto groundwater study area. Biogeochemical hotspots commonly occur at the boundaries or ecotones between two features in a landscape (McClain et al., 2003). Indeed, the denitrification hotspot characterized by Hosono et al. (2013) was located at a hill–plain border area. Furthermore, anaerobic autotrophic denitrifiers and methanogens were detected in the denitrification hotspot using $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ tracers (Hosono et al., 2014). Although many studies have analyzed denitrification hotspots and their microbial communities, to authors' knowledge, the profile of microbial community including denitrifiers in the Kumamoto groundwater area is still poorly understood. Basic knowledge regarding the microbial community and the taxa involved in the denitrification process is urgently needed.

Therefore, the objective of this study was to compare the microbial communities inside and outside of the denitrification hotspot using a cloning-library method and phylogenetic analysis to explain the occurrence of the denitrification hotspot. The results obtained in the present work provide additional information regarding the distribution of denitrifiers in regions of confined groundwater and further the mechanism of denitrification in the Kumamoto groundwater studying area.

2. Materials and Method

2.1 Sampling sites

The Kumamoto groundwater studying area, located at approximately 32.58N to 32.92N and 130.6E to 131.0E, was defined as the area surrounded by the divide of Mount Aso (1592 m) to the east, the Midori River to the south, the Ariake Sea and Mount Kinpo to the west, and the Kikuchi River to the north (Hosono et al., 2013). This area includes two main groundwater flow lines: A-A' and B-B'. Two and five confined groundwater samples along the A-A' and B-B' lines, respectively, were collected from this area, as shown in Fig. 1, between August and November 2013. Samples B-I, B-T, and B-S were obtained from zone B2, where the denitrification hotspot was detected. Outside of the denitrification hotspot, samples B-Y and B-H were obtained in the recharge zone B1, and samples A-F and A-W were obtained from zone A2 along line A-A'; these specimens were used as control samples in the analysis.

2.2 Measurement of hydrochemical parameters

During sample collection, physicochemical parameters, such as depth, pH, dissolved oxygen (DO), and oxidation-reduction potential (ORP), were directly measured in the field using handheld meters. In the lab, the concentration of dissolved organic carbon (DOC) was detected using TOC-VWS analyzer (Shimadzu, Japan) with the non-purgeable organic carbon method. Samples were acidified to below pH 3, providing the removal of inorganic carbon (IC) as CO_2 . The DOC then was determined by subtracting the IC value from the Total Carbon (TC) measurement. Additionally, NO_3 and Cl were measured by ion chromatography (Compact IC 761, Metrohm, Switzerland) using standard solutions; this technique had a detection limitation below 0.05 mg l^{-1} . Approximately 10 l of each water sample were filtered through the 0.22- μm Sterivex sterile filter unit (Millipore, Billerica, MA, USA) to obtain microorganisms for DNA extraction.

2.3 DNA extraction and cloning-library analysis

The total DNA from seven filtered samples was extracted using the PowerWater SterivexTM DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, PCR amplification was performed. The primer sets for PCR and corresponding mixture and conditions during amplification were shown in

the supplementary Table S1. The purified PCR products were inserted into the pT7 Blue Vector (Novagen, Cambridge, MA, USA) and transferred into competent cell *Escherichia coli* DH5 α according to the manufacturer's protocols. After transformation, plasmid extraction was subsequently performed using the Wizard SV Minipreps DNA Purification System (Promega, Madison, WI, USA). Both *EcoRI* and *PstI* restriction enzymes were used to identify the positive clones as specified by the manufacturer. Following sequencing and chimera-checking (Edgar et al., 2011), operational taxonomic unit (OTU) classification was performed with Mothur (Schloss et al., 2009) and the nearest strains of the representative OTU clones were searched in NCBI database. Finally, the phylogenetic tree was built using MEGA 5.0 with the neighbor-joining method (Tamura et al., 2011).

2.4 Accession numbers

The bacterial, archaeal and *nirS* sequences used to detect the OTUs in this work were submitted to the DNA Data Bank of Japan (DDBJ) as accession numbers LC082102–LC082131.

3. Results and Discussion

3.1 Characteristics of groundwater samples

The hydrochemical parameters of the seven samples are summarized in Table 1. Along A-A', the concentration of O₂ and ORP decreased from more than 2.0 mg l⁻¹ and 200 mV in samples (n=2) upstream of the hotspot to less than 1 mg l⁻¹ and negative in samples (n=3) inside of the hotspot respectively. The changing tendency was consistent with a previous study (Li et al., 2011). Additionally, the DOC concentration increased abruptly from 0.4 to 2.07 mg l⁻¹ while the Cl⁻ concentration decrease from 64.4 to 4.4 mg l⁻¹ between the B1 and B2 zones, possibly due to the infiltration of freshwater with DOC from the surface water. The subsequent decrease of DOC, often used as a carbon source and electron donor, from upstream to downstream indicated stimulation of heterotrophic bacteria including denitrifiers and competition between those organisms in the hotspot (Zhang et al., 2016). The concentration of NO₃ fell below detectable limits in the B2 zone; depletion of nitrate confirmed the existence of the denitrification hotspot. Conversely, the NO₃ concentrations in the A2 zone increased when compared samples A-F and A-W (10.3 mg l⁻¹ and 19.9 mg l⁻¹, respectively). The high ORP and DO concentration detected in A-W indicated that processes at this site were strictly aerobic.

3.2 Phylogenetic analysis of bacterial 16S rDNA

A total of 199 clones, including 20, 18, 30, 32, 31, 33, and 35 clones obtained from samples A-F, A-W, B-Y, B-H, B-I, B-T, and B-S, respectively, were subjected to cloning analysis after chimera checking and classified into 59 OTUs (similarity >97%). The main OTUs defined as those represented by more than three clones corresponded to 138 clones in total; these OTUs were used to build a phylogenetic tree (Fig. 2). These clones clustered into five groups: *α -Proteobacteria*, *β -Proteobacteria*, *γ -Proteobacteria*, *Firmicutes* and *Actinobacteria*. The most numerous bacteria in the present study were *Proteobacteria*, which was consistent with previous studies characterizing microbial communities in deep bedrock (Larentis et al., 2015), and in both confined and unconfined aquifer ecosystems located under a dairy farming region (Smith et al., 2012).

In the class *α -Proteobacteria*, LC082107 was the largest group consisting of 31 and 23 clones from the B1 and B2 zones, respectively, and showed 99% similarity to *Methylobacterium mesophilicum*. This organism, originally isolated from the pink film in a bathroom, is a gram-negative, strictly aerobic, pink-pigmented, facultative methylotrophic bacterium (Yano et al., 2013). This type II methylotrophic bacterium can utilize methanol,

formate, and other C1 sources via the serine pathway. In the γ -Proteobacteria cluster, LC082102 and LC082104 consisting of 4 and 11 clones both from the B2 zone displayed 98% and 91% identity to *Methylomonas rubra* and *M. paludis*, respectively. *M. paludis* is an aerobic methylotrophic bacterium, originally isolated from an acidic (pH 3.9) *Sphagnum* peat bog in northeastern Russia, possesses a particular methane monooxygenase enzyme and utilizes only methane and methanol (Danilova et al., 2013). The nearest strain of LC082103 owing five clones from the B2 zone was *Methylobacter psychrophilus*. Both *Methylomonas* and *Methylobacter* are type I methylotrophic bacteria that can utilize C1 compounds via the ribulose monophosphate pathway. Although the B1 and B2 zones are both suboxic environments, no clones corresponding to anaerobic methanotrophic archaea were found in the present study, and the diversity of methanogens was lower than that observed in a previous study (Zhou et al., 2015). The growth of type II methylotrophs is favored under low-oxygen, high-methane conditions, and type I methylotrophs outcompete type II methylotrophs at low-methane concentrations (Graham et al., 1993). However, the wide distribution of the *Methylobacterium* spp. in the B1 and B2 zones where have wide ranges of DO and DOC suggested that these type II methylotrophs easily adapt to changing environments, a hypothesis that is not consistent with previous findings.

Among the class β -Proteobacteria, LC082106 owing five clones was associated with *Sulfuritalea hydrogenivorans* sk43H with 99% identity. This strain was isolated from freshwater lake sediments in Japan; although this organism originally was characterized as a facultative anaerobe, it was still found in sample A-W with DO concentration of 8.08 mg l⁻¹ (Table 1). *S. hydrogenivorans* sk43H can oxidize thiosulfate, elemental sulfur, or hydrogen as a sole energy sources for autotrophic growth, alternatively, this bacterium can oxidize acetate, benzoate, pyruvate, and succinate for heterotrophic growth, utilizing nitrate as an electron acceptor (Kojima and Fukui, 2010). The closest strain to representative LC082111 was *Bacillus subtilis* in the phylum Firmicutes, with 100% identity. Some *B. subtilis* strains have been isolated from ammonia-rich wastewater and are able to directly covert ammonia to gaseous nitrogen under aerobic conditions (Kim et al., 2005; Yang et al., 2011). Gram-positive bacteria, including *Bacillus*, have been shown to be involved in the denitrifying process in wastewater treatment systems and paddy soil (Verbaendert et al., 2011).

3.3 Distribution of bacterial 16S rDNA

The distribution of the main genus and family in each zone is shown in Fig. 3. Methylotrophs, Firmicutes, and *Corynebacterium* were distributed in both B1 and B2, along the B-B' line. However, members of the genera *Curvibacter* and *Sulfuritalea* which belong to the class β -Proteobacteria, were found primarily in the A2 zone. Interestingly, type I methylotrophic bacteria, such as *Methylobacter* spp. and *Methylomonas* spp., the family Methylococcaceae only inhabited the B2 zone. Conversely, another type II methylotroph, genus methylocystis was found in the A2 zone, though this organism was apparently less abundant (i.e., represented by fewer clones). In addition, it was clear that the bacterial diversity of the B2 zone was much higher than that of the other two zones. Notably, among the other genera, despite low clone numbers, the sequences closely related to denitrifiers such as *Pseudomonas stutzeri* in sample B-Y and *Sideroxydans lithotrophicus* in sample B-I were found in the present study.

3.4 Phylogenetic tree of archaeal 16S rDNA

After chimera checking, 144 non-chimeric archaeal sequences was isolated, including 22, 16, 21, 15, 22, 25, and 23 clones from samples A-F, A-W, B-Y, B-H, B-I, B-T, and B-S, respectively. These sequences were classified into 50 OTUs with the cutoff of 0.03. Thirteen major OTUs with more than two clones were selected to build the phylogenetic tree after

identified the closest known strains in BLAST. As shown in the supplementary Fig. S1, these sequences grouped into two clusters (phyla): *Euryarchaeota* and *Thaumarchaeota*.

Representative LC082112 with two and five clones found in the B1 and B2 zones, respectively, were associated with *Methanobacterium* sp. SA-12 with 99% identity. Likewise, LC082113 and LC082114 showed 98% and 99% identity to known archaea *M. aarhusense* strain H2-LR and *M. flexile* strain GH, respectively. Methane-producing archaea utilize H_2/CO_2 or acetate to produce methane in the strictly anaerobic environments (Thauer, 1998), where the methane then is oxidized by methylotrophic bacteria. The cloning-library analysis suggested that the ratios of methane-producing archaeal clones to total archaeal clones were 12%, 63%, and 0% in zones B1, B2, and A2, respectively, which appeared to correlate with the distribution of methane-assimilating bacteria such as *Methylobacter* and *Methylomonas*. Previous isotopic analysis provided evidence for the occurrence of the methane-producing reaction in the B2 zone (Hosono et al., 2014), and this interpretation is supported by the distribution of *Methanobacterium* in the B2 zone in the present study.

Members of *Thaumarchaeota*, another archaeal phylum, also were obtained during analysis of archaeal 16S rDNA. A total of 32 clones belonging to *Thaumarchaeota* were primarily found the B1 and A2 zones, where the DO concentration was relatively high. LC082120 and LC082121 displayed 96% and 90% identity to *Candidatus Nitrososphaera* sp. EN76, respectively. This ammonia-oxidizing archaeon (AOA) originally was isolated from soil and can utilize ammonia or urea as an energy source; the organism is capable of growth at high ammonia concentrations (Tourna et al., 2011). LC082123 and LC082124 enhancing 3 and 16 clones showed 93% and 100% identity to *Candidatus Nitrosotalea devanatterra* isolate Nd1, respectively. Isolated and cultivated from acidic agricultural soil, this archaeon was involved in the ammonia oxidization process (Lehtovirta-Morley et al., 2011; 2014). Intriguingly, though originally identified as an acidophilic archaeon, *Candidatus N. devanatterra* was detected in the present study in confined groundwater where is neutral environment. Previous studies have demonstrated that AOA inhabit not only soil (Prosser and Nicol, 2012), but also groundwater systems (Rogers and Casciotti, 2010), and that AOA play more important roles in the nitrification process than ammonia-oxidizing bacteria (Prosser and Nicol, 2008; Rogers and Casciotti, 2010). However, few studies have reported the detection of acidophilic archaea *Candidatus N. devanatterra* in the neutral groundwater environments. The occurrence of this organism in confined groundwater remains to be further characterized in future studies.

3.5 Phylogenetic analysis of *nirS*

A total of 59 *nirS*-positive clones were obtained, including 3, 3, 19, 20, and 14 clones from sample B-Y, B-I, B-T, B-S and A-F, respectively. The major OTUs as well as their closest strains are summarized in the supplementary Table S2. These *nirS*-related sequences showed 77–99% identity to the reference strains. Consistent with previous studies (Iribar et al., 2008; Katsuyama et al., 2013), all *nirS*-related sequences were derived from known denitrifying *Proteobacteria* except for the uncultured and poorly characterized bacterial strain designated “Clone J16”.

LC082127 was associated with *Sulfuricella denitrificans* skB26 with 82% identity. *S. denitrificans* skB26, isolated from anoxic freshwater lake sediment in Japan, is a novel facultative anaerobic sulfur-oxidizing bacterium. This organism can oxidize elemental sulfur or thiosulfate to sulfate under micro-aerobic conditions utilizing nitrate as an electron acceptor during reduction of nitrate to nitrogen (Kojima and Fukui, 2010). Similarly, LC082129 showed 94% identity to *Sulfuritalea hydrogenivorans* sk43H. This strain was also isolated from freshwater lake sediment in Japan, and is a facultative anaerobic bacterium that exhibits autotrophic growth only under anaerobic conditions. The isolate can use thiosulfate, elemental sulfur or hydrogen as a sole energy sources for autotrophic growth with nitrate as

an electron acceptor (Kojima and Fukui, 2011). The nearest strain of LC082130 was *Pseudomonas balearica* DSM 6083 originally isolated from sewage water with 99% identity. This organism can oxidize inorganic sulfur or thiosulfate compounds to tetrathionate under denitrifying conditions using NO₃, NO₂, or N₂O as an electron acceptor (Sorokin et al., 1999). LC082131 including 28 clones accounted for about half of all *nirS*-related sequences obtained in the present study. The closest strain of LC082131 was the uncultured bacterium clone J16 (JF966879.1) with 99% identity. Though little is known about this denitrifying bacterium, this sequence was detected in both the B1 and B2 zones in the present study. Therefore, It was speculated that this type of denitrifier plays an important role in the depletion of nitrate within the denitrification hotspot. However, the 16S rDNA and *nirS* analysis suggested the presence of large numbers of denitrifiers in sample A-F with relatively low level of DO and ORP and elevated level of NO₃. Comparison of the denitrifier species and bacterial community structure between samples A-F and B-S is expected to clarify the occurrence and activity of the denitrification process at this study's hotspot.

4. Conclusions

The main purpose of this study was to characterize, using hydrochemical data and microbial diversity and taxon distributions, the microbial community associated with biotic denitrification process in confined groundwater. Although relatively few clones were obtained in this study despite repeated attempts and lack of quantitative data, the obtained cloning data provide an approximation of the microbial community profile inside and outside of the denitrification hotspot. Notably, kinds of denitrifiers were detected including autotrophic and heterotrophic denitrifier. Additionally, methane cycle-associated methylotrophs and methanogens were detected in the denitrification hotspot along with these denitrifiers. Therefore, it was speculated that active denitrification with the methane cycling occurs under anaerobic conditions, and that this biotic process is maintained by continuous DOC contamination in the B2 zone. Interestingly, AOA were found in the neutral confined aquifer environment. The comparative analysis of the microbial distribution and corresponding environment variables in each zone provided useful knowledge about microorganisms, including denitrifiers, involved in nitrate reduction in Kumamoto groundwater. Investigation of denitrifier activity between these denitrifiers and isolation and enrichment of AOA will be performed in the near future.

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Table 1 Hydrochemical parameters for seven deep groundwater samples obtained from Kumamoto **groundwater** studying area

Zone	Sample name	Longitude (E)	Latitude (N)	Well depth (m)	pH	DO (mg l ⁻¹)	DOC (mg l ⁻¹)	ORP (mV)	NO ₃ (mg l ⁻¹)	Cl (mg l ⁻¹)
A2	A-F	130.82166	32.82062	90	7.43	2.37	1.29	65	10.3	9.8
	A-W	130.81228	32.81715	70	7.24	8.08	5.36	342	19.9	9.2
B1	B-Y	130.71992	32.84569	124	6.45	3.17	0.75	221	0.8	34.9
	B-H	130.72133	32.84175	131	6.87	2.31	0.4	266	2.4	64.4
B2	B-T	130.71411	32.81795	50	7.22	0.83	2.07	-70	0	4.4
	B-1	130.69850	32.82118	91	7.94	0.1	0.3	-261	0	3.8
	B-S	130.71570	32.80015	65	7.67	0.6	0.59	-199	0	15.2

List of figure captions

Fig. 1 Sampling sites in the Kumamoto groundwater study area.

Fig. 2 Phylogenetic analysis of the main operational taxonomic units (OTUs), defined as those represented by more than three bacterial 16S rDNA clones.

The strains most closely related to each clone were identified by BLAST searches; uncultured/environmental sample were excluded. Both the retrieved clones and their closely related isolates in the BLAST search were used to build a phylogenetic tree with the neighbor-joining method. The zone name and number in parentheses next to the accession number indicate the number of clones obtained in each zone. The numbers at the nodes show the confidence levels of the clustering based on 1000 bootstrap replicates

Fig. 3 Distribution of the main bacterial genera and family to which the clones affiliated in each zone.

Supplementary Fig. S1 Phylogenetic analysis of the main OTUs, defined as those represented by more than two archaeal 16S rDNA clones.

The strains most closely related to each of the obtained clones were identified by BLAST searches; uncultured/environmental sample were excluded. Both the retrieved clones and their closest clones in the BLAST analysis were used to build a phylogenetic tree with the neighbor-joining method. The zone name and number in parentheses near the accession number indicate the number of clones obtained from each zone. Numbers at the nodes show the confidence levels of the clustering based on 1000 bootstrap replicates.

Fig 1

Fig 2

Fig 3

Confined Groundwater Samples

Graphical abstract