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Short communication

Comparison of microbial communities inside and outside of a denitrification hotspot 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.

1. Introduction

Nitrate (NO_3) 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 (N_2O or N_2) 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 NO_3 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 NO_3 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

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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 $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ 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–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 Sterivex™ 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 (Weisburg et al., 1991; Jurgens et al., 1997; Braker et al., 1998). The purified PCR products were inserted into the pT7 Blue Vector (Novagen, Cambridge, MA, USA) and transferred into competent cell *Escherichia coli* DH5α 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 *EcoR*I and *Pst*I 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 O_2 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 NO_3 fell below detectable limits in the B2 zone; depletion of nitrate confirmed the existence of the denitrification hotspot. Conversely, the NO_3 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