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Relationships between Microbial Compositions and Hydrochemical Factors in Nitrate Contaminated Groundwater of Hun River Alluvial Proluvial Fan, China

A. X. Zhou[1, 2], Y. L. Zhang[1, 2*], J. Y. Dang[1, 2], and X. S. Su[1, 2]

ABSTRACT

The objective of this study was to explore the microbial community diversities and the relationships between microbial community compositions and hydrochemical factors in nitrate contaminated groundwater of Hun River alluvial plain. The method of polymerase chain reaction (PCR)-denaturing, gradient gel electrophoresis (DGGE) gene fingerprints combined with canonical correspondence analysis (CCA) were applied. The Operational Taxonomic Units (OTUs) of all the sampling sites had a certain degree of heteroplasmy and 75% OTUs presented in less than half of the sampling sites. The un-weighted pair group mean average (UPGMA) cluster analysis showed that the microbial community similarity of all the sampling sites were not relatively high (0.6-0.8). The distribution of microbial community positively correlated with nitrate. The dominant bacteria of the nitrate contaminated groundwater mainly included *Hyphomicrobium denitrifican* sp., *Halanaerobium praevalens* sp., *Desulfotomaculum reducens* sp., *Nitrosospira multiformis* sp., among which *the Nitrosospira multiformis* sp. and *Sulfurovum* sp. existed in all the sampling sites. CCA results indicated that $Mn^{2+}$and NO_3^- were the most relevant hydrochemical factors to regulate the microbial composition in nitrate contaminated groundwater of this area, and next were Fe^{2+} and SO_4^{2-}. The results could provide references for the bioremediation of the nitrate contaminated groundwater of Hun River alluvial plain

Keywords: Bacteria, Bioremediation, PCR-denaturing.

INTRODUCTION

Nitrate, one of the most common pollutants in groundwater, causes a significant water-quality issue worldwide, especially in agricultural regions (Javadi *et al*., 2011). Nitrate originates from various anthropogenic sources, such as fertilizers, animal manure, domestic waste water and septic tanks, as well as organic nitrogen from soil (Min *et al.*, 2002; Lee *et al.*, 2008; Staver and Brinsfield, 1998). Excess nitrates in the drinking water cause health risks such as methemoglobinemia - "blue baby" disorder (Weyer *et al*,. 2001; Fewtrell, 2004; Ye *et al.*, 2010).

Denitrification is the most significant process removing nitrate in natural environments. But, denitrification in natural systems proceeds very slowly and is not very effective in lowering nitrate concentrations in aquifers. This is why several technologies have been developed for removing nitrate (Della Rocca *et al*., 2007). A conceivable bioremediation strategy is biostimulation of the indigenous denitrifying bacteria by addition of suitable electron donors (Soares, 2000). This strategy requires the existence of an indigenous microbial community able to

[1] Key Laboratory of Groundwater Resources and Environments, Ministry of Education, Jilin University, Changchun, People's Republic of China.
[2] Institute of Water Resources and Environment, Jilin University, Changchun, People's Republic of China.
[*] Corresponding author; email: lingling29@126.com

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reduce nitrate using the added electron donor. Before applying this bioremediation strategy in the field, it is also necessary to evaluate which electron donors are present in the groundwater of this area that can be utilized by the indigenous microorganisms to reduce nitrate. However, 99% of microorganisms are uncultured (Tomaru *et al.*, 2014). Utilizing the traditional techniques could not fulfill our research needs, but the microbial molecular ecological technologies such as polymerase chain reaction -denaturing gradient gel electrophoresis (PCR-DGGE) provides possibilities to study the microbial community (Flynn *et al.*, 2000; Xing *et al.*, 2006).

Denitrification is a redox reaction driven by autotrophic or heterotrophic bacteria that reduce NO_3^- to nitrogen gas (N_2) under suboxic conditions. Autotrophic bacteria promote denitrification using reduced sulfur, Fe^{2+}, Mn^{2+} compounds (Tartakovsky *et al.*, 2002; Khan and Spalding, 2004; Vidal-Gavilan *et al.*, 2013). Heterotrophic denitrification occurs through a number of sequential reactions where bacteria use organic matter as the electron donors for NO_3^- reduction. In both processes, NO_3^- is initially converted to nitrite (NO_2^-), which is more toxic than NO_3^-(De Beer *et al.*, 1997); The next reaction transforms NO_2^- into nitric oxide gas (NO), and NO is subsequently converted into nitrous oxide gas (N_2O); both species are greenhouse gases. Finally, N_2O is transformed into N_2. Before any field application, a detailed in-situ characterization is required. Main electron donors existing in the groundwater should be clear. If the biostimulation is based on the initial hydrochemical factors, the adaptive phase of the indigenous microbial community will be shortened, promoting the reduction process and decreasing toxic intermediate products. CCA, a direct gradient analysis, was used to examine the relationship between bacterial community profiles and environmental factors (Andrushchyshyn *et al.*, 2009). CCA has been shown to provide a flexible and meaningful constrained ordination of ecological species abundance data with environmental variables(Anderson and Willis, 2003). CCA analysis results would be helpful to select the most significant environmental factors (Mou *et al.*, 2010).

The main purposes of this study were: (a) to investigate the microorganism diversity in the nitrate contaminated groundwater, and (b) to determine the relationships between spatial microbial community composition and groundwater hydrochemical factors. The results provide references for the bioremediation of the nitrate contaminated groundwater of Hun River alluvial plain.

MATERIALS AND METHODS

Study Area

Hun River originated from the north face of Gunmaling Qingyuan county Fushun city, flowing from northeast to southwest, mainly through Fushun city and Shenyang city. The length of this river is 415 km (1.36×10^6 ft), and the river mouth was an enlargement area, developing to Hun river alluvial proluvial Fan. Hun River receives sanitary sewage of Fushun city and Shenyang city and industrial wastewater from petrochemical processing, metallurgy, machinery, and light industry. In history, this area had made use of wastewater from Hun River to irrigate the 56×10^4 hectare farmland, which had caused serious influences on the farmland soil, crops, and groundwater, especially the groundwater contaminated with nitrate.

Sample Collection

A total of 12 wells were investigated. Their locations and the land use types surrounding the wells are presented in Figure 1. There were six wells in the urban region, two wells at the vicinity of industrial region, and four wells in the farm land region. The samples were taken using sterile bottles in August and early September, 2012. According to different testing targets, protective agents were added to the groundwater samples to avoid the test

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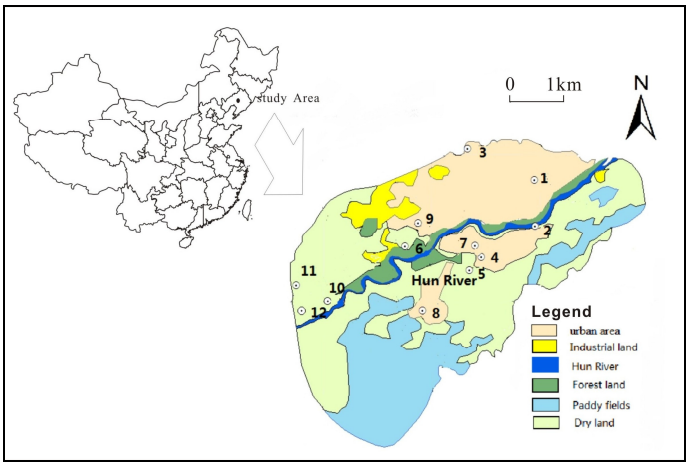

Figure 1. Distribution of the groundwater sampling sites of Hun River alluvial plain, and the land use types in this area.

elements volatilizing or being oxidized or reduced and prevent other physicochemical reactions (Table 1). All the samples were triplicate. The groundwater samples were kept at -4°C in the dark and carried back to laboratory for analysis as soon as possible.

PCR Amplification and DGGE

Three liters of groundwater were collected to filtrate through membranes pore size of 0.22 microns for DNA extraction. Total genomic DNA extraction was performed as previously described (Ferreira and Martin-Didonet, 2012). The primer combination of GC-F338 /R518 was used to amplify the variable regions (V3) of 16S rDNA of the microbial community (Liu *et al.*, 2007). PCR reactions were performed in a Stratagene Mx 3000P Thermal Cycler (Stratagene Laboratories, USA). DGGE was performed at 60°C with D-CodeTM Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. Thirty μL of the PCR products were applied on a DGGE gel of 8% polyacrylamide with a linear denaturing gradient ranging from 30 to 60% (100% denaturing gradient contains 40% formamide and 42% urea). Electrophoresis

Table 1. The added protective agents and analysis methods.

Testing targets	Protective agents	Analysis methods
NO_2^- NO_3^- SO_4^{2-}	No protective agents	Ion Chromatography (LC-20A, Shimadzu co. Japan)
DO	No protective agents	Portable analyzer (HACH, USA)
Fe^{2+}	Add 1 mL 1:1 H_2SO_4 and 0.1g $(NH_4)_2SO_4$ in 10 mL groundwater	Voltammetric trace analysis instrument (797 VA Computrace, Metrohm co., Switzerland)
Fe^{3+}	Add 2 mL hydrochloric acid per 100 mL groundwater	
Mn^{2+}	Add HNO_3 to the pH1~2	
NH_4^+	Add 0.8 mL concentrated sulfuric acid per 1L groundwater	Methods of oxidized by hypobromite and molybdophosphoric blue

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was run at a constant voltage of 120V for 400 min in 1×TAE buffer. Subsequently, the gels were stained with ethidium bromide (0.5 mg L^{-1}) for 30 minutes and gel digital images were obtained using UV transilluminator (MiniBIS Pro, DNR).

The DGGE profiles were analyzed with the Quantity One 4.6.2 program. Bands with intensity < 0.06 were excluded from the analysis. A dendrogram between all lanes was obtained by UPGMA cluster analysis. We used the Shannon-Weaver's index (H) as a measure of species diversity (Shannon and Weaver, 1949), and the Simpson index J to measure the equitability/dominance (Simpson, 1949).

Sequencing and Nucleotide Sequence

The DNA fragments were recovered from the DGGE gel by the EZ Spin Column PAGE Gel DNA Extraction kit (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.). Two **µL** of eluted DNA were used as the templates for amplification with the primers GC-F338 and R518. The original and the amplified DNA were performed together rerun in a DGGE gel to check the accuracy of the process. Bands of DNA were re-excised and treated as described above when it was necessary. Sequencing was finished by Shanghai Sangon and sequencing reactions were run on an ABI 3730 apparatus. The obtained partial 16S rDNA sequences of bacteria were subjected to a NCBI BLASTN (http://www.ncbi.nlm.gov/blast/) search to identify sequences with highest similarity. The sequences were then grouped with relevant reference sequences obtained from GeneBank and multiplied aligned using Clustal W.

Canonical Correspondence Analysis (CCA)

In DGGE profile, each band was related to one single population and considered to be one operational taxonomic unit (OTU). Co-migration points of DGGE profiles were used to build a matrix based on the relative OTU intensity in each lane after log transformation. Canonical correspondence analysis (CCA), originally developed to relate plankton community composition to hydrochemical factors (Yan *et al.*, 2008), was used here to investigate the relationships between bacteria community compositions and hydrochemical factors, and CCA was performed using the software program CANOCO 4.5 (Jongman,1997). Additionally, the hydrochemical factors with high partial correlation coefficients (P> 0.05) were eliminated from the final CCA. To satisfy the assumption of normality and homogeneity of variance in the data, all data were logarithmically transformed before the analyses.

RESULTS

Hydrogeological Factors

The temperature of groundwater was about 10°C and pH ranged from 5.6 to 8.3, most of sites below 7, presenting weak acidic. The total distribution of nitrate was conformed to different land use types (Figure 2) (Hosseini *et al.*, 2012). The peak value of nitrate appeared in northwest part of this field. The main land use types here were industrial and urban areas, and the groundwater nitrate were from industrial and sewage water leakage (Torrentó *et al.*, 2011). Additionally, in history, this area made use of wastewater from Hun River to irrigate, which also carried numerous nitrate contaminations to the groundwater. Second peak value of nitrate appeared in southeast area of this field. The land use type of this area was farm land and, because of applying excessive nitrogenous fertilizer to the soil, the groundwater was polluted by nitrate. All in all, the main nitrate source of this area is industrial and sewage water leakage and nitrogenous fertilizer. The nitrate concentration in most parts of this field exceeded the Drinking Water Standards and Health Advisories' (EPA) maximum

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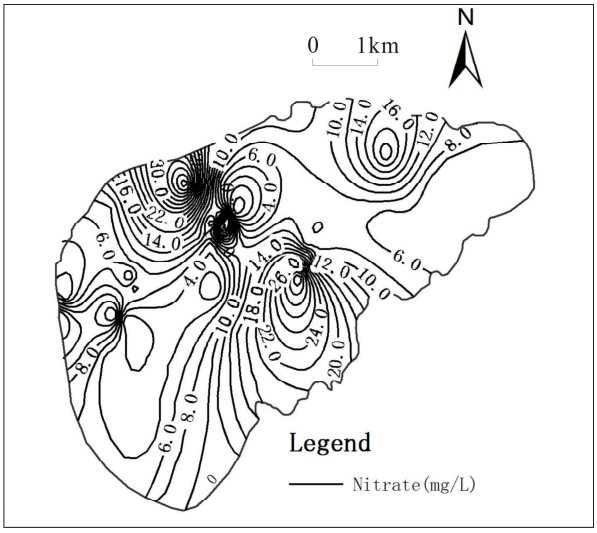

Figure 2. Distribution of the nitrate concentration of groundwater in Hun River alluvial plain.

contaminant level (10 mg NL^{-1}). The highest value of nitrate in the groundwater of this region was 30 mg L^{-1}, higher two times than the maximum contaminant level.

DGGE Profiles Analysis

The results of DGGE are shown in Figure 3, and every red line represented each bacterial OTU. Twenty different 16Sr DNA OTUs were detected in the DGGE analysis. Six of the 20 bacterial OTUs (30%) were site-specific, and only five were common to all the investigated samples. Additionally, 75% of the bacterial OTUs were detected in less than half of the samples. The similarities of the microbial community composition between all the sampling sites, as visualized by UPGMA clustering, were not high (0.47-0.80). These results indicated that the microbial community composition in groundwater of this region had heterogeneity to a certain extent among the sampling sites.

The distribution of Shannon-Weaver's index and Simpson index (D) in this study area are shown in Figure 4. The highest value of Shannon-Weiner index appeared in northeast area of the field, the upstream of the groundwater. The reason was that nitrate reduced the microbial community diversity and increased the dominance (Simpson index). The community that could utilize the nitrate tended to be dominant, as also demonstrated by Torrento *et al.* (2011). The rapid response of the indigenous bacterial community was to adapt to the new conditions and efficiently reduce nitrate (Torrentó, *et al.*, 2010). From Figures 2 and 4, the distribution of the nitrate concentration of groundwater showed certain similarity with the distribution of Shannon-Weaver's (H) index and Simpson index (D) in Hun River alluvial plain. As a result, the diversity of community in the nitrate contaminated groundwater negatively correlated with the concentration of nitrate.

Sequencing of DGGE OTUs

The dominant strains in this study region included Hyphomicrobium denitrifican sp., Halanaerobium praevalens sp., Desulfotomaculum reducens sp., Nitrosospira multiformis sp. Sulfurovum sp. and Thiobacillus denitrificans sp. (Table 2). In fact, in earlier studies, species belonging or closely related to these groups have been

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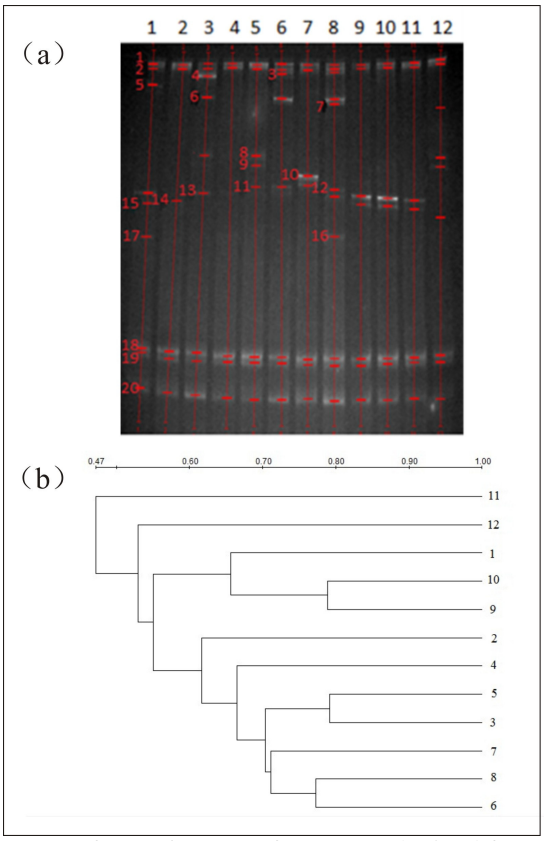

Figure 3. (a) DGGE patterns of V3-16S rDNA fragments obtained from the studied area, and (b) UPMGA cluster analysis of DGGE profile.

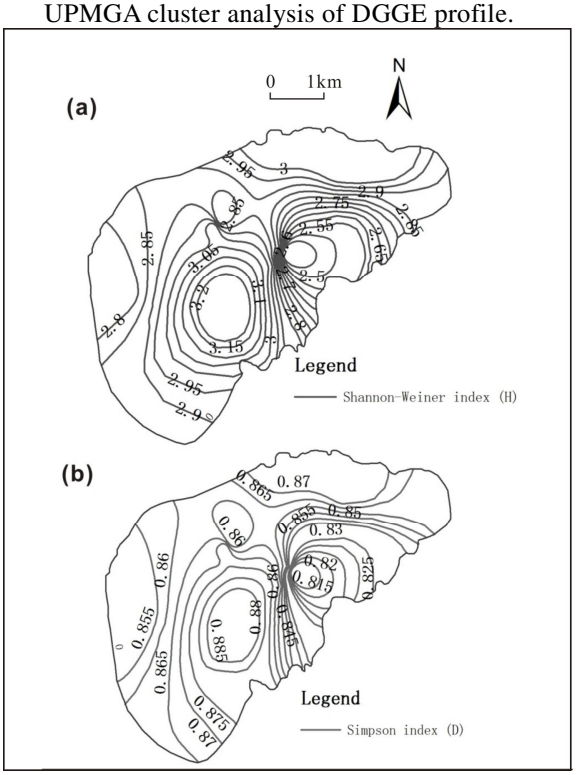

Figure 4. The distribution of (a) Shannon-Weaver's (H) index, and (b) Simpson index (D) in the study area.

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Table 2. Closest matches to excised and sequenced 16S rDNA derived from DGGE OTUs.

OTU	Closest relative	Accession no.	Similarity (%)
2	*Bacillus subtilis* sp.	NC_014976.1	100
4	*Hyphomicrobium denitrificans* sp.	NC_014313.1	96
6	*Halanaerobium praevalens* sp.	NC_017455.1	92
5	*Dehalogenimonas* sp.	NC_009253.1	89
7	*Desulfotomaculum reducens* sp.	NC_009253.1	86
8	*Pseudovibrio* sp.	NC_016642.	93
10	*Thiobacillus denitrificans* sp.	NC_007614.1	92
13	*Methylocella silvestris* sp.	NC_011666.1	96
18	*Nitrosospira multiformis* sp.	NC_00764.1	92
20	*Sulfurovum* sp.	NC_009663.1	89

reported as denitrifying bacteria (Finkmann et al., 2000; Ginige et al., 2004; Osaka et al., 2006; Cardenas et al., 2008; Sahu et al., 2009; Sun et al., 2009).

Shi *et al*. (2013) reported a nitrate-uptake bacterial strain bacillus in the nitrate contaminated soil. In our study, OTU-2 (100% to *bacillus subtilis* sp.) related to bacillus was found, which may play an important role in the nitrate reduction using Mn^{2+} as electron donor. OTU-4 (96% related to *Hyphomicrobium denitrifican* sp.) and OTU-10 (92% related to *Thiobacillus denitrificans* sp.) could utilize ferrous and sulfur as electron donors in the process of nitrate reduction, as reported in the previous studies (Baytshtok *et al.*, 2008; Haaijer *et al.*, 2008; Wang *et al.*, 2014). OTU-7(86% to *Desulfotomaculum reducens* sp.) and OTU-20 (89% to *Sulfurovum* sp.) are the species related to sulfate reduction and nitrate reduction simultaneously (Haaijer *et al.*, 2008). OTU-18 (92% to *Nitrosospira multiformis* sp.) existed in all the samples and was responsible for oxidizing ammonia to nitrous acid (Haaijer *et al.*, 2008).

CCA Analysis

CCA analyses were performed using the selected hydrochemical variables with bacterial OTU composition. The first two CCA ordination axes explained 41.4% of the bacterial OTU variation. The 51.9% of the cumulative variance of OTU-environment relation was represented by the first two axes (Table 3).The CCA ordinations showed

Table 3. Summary results of the canonical correspondence.

Parameter	Axis Ⅰ	Axis Ⅱ
DO	-0.1137	0.1471
SO_4^{2-} (mg L^{-1})	-0.4249	0.2107
NO_3^- (mg L^{-1})	0.3002	0.4675
NO_2^- (mg L^{-1})	-0.0024	0.3627
NH_4^+ (mg L^{-1})	-0.0670	-0.4334
Fe^{2+} (mg L^{-1})	-0.2902	0.1162
Mn^{2+} (mg L^{-1})	0.8114	-0.3695
Fe^{3+}(mg/L)	-0.0856	-0.1234
Eigenvalues	0.345	0.317
OTU-environment correlations	0.999	0.993
*CPV of OTU data	21.6	41.4
*CPV of OTU-environment relation	27.0	51.9

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that the distribution of bacterial OTUs was primarily correlated with selected hydrochemical variables Figure 5 . Briefly, SO_4^{2-} and Fe^{2+} were negatively (r= -0.4249, r= -0.2902) correlated with the first CCA axis, while Mn^{2+} was positively(r= 0.8114) correlated with the first CCA axis. Both NO_3^- (r= 0.4675) and NO_2^- (r= 0.3627) were positively correlated with the second CCA axis, while NH_4^+ (r= -0.4334) were negatively correlated with the second CCA axis.

The CCA ordinations showed the relationship between distribution of bacterial OTUs and hydrochemistry variables Figure 5 . In the ordination diagrams, the arrow length of the environmental variables indicate the degree of influence on the microbial community, and the subpoint of the species on the environmental variable arrowheads or their extension lines could indicate their correlation. The original point indicates the correlation average value. From Figure 5, the environmental variables significantly affected by the microbial community are NO_3^- and Mn^{2+}. For example, OTU-4 (96% similar to *Hyphomicrobium denitrifican* sp.) and OTU-7 (86% to *Desulfotomaculum reducens* sp.), OTU-4 is negatively correlated with NO_3^-, while OTU-7 is positively correlated with NO_3^-. We also find that a great majority of the species is positively correlated with the NO_3^-, and distributed in the second quadrant. Mn^{2+} is the main electron donor in the groundwater, the next is Fe^{2+}.

DISCUSSION

Effect of Land Use Types

In this area, the sewage water leakage and the nitrogenous fertilizers were the main sources for the groundwater nitrate. In previous studies, the overuse of nitrogen (N) fertilizer also had been identified as the main source of groundwater nitrate in the other areas (Li *et al.*, 2007; Kaushal *et al.*, 2011). Meanwhile, studies by Gu *et al.* (2011; 2012) showed that groundwater nitrate could be from sewer leakage and landfill leachate, which are of growing importance alongside urbanization. However, considerable uncertainty remain in our knowledge of the magnitude and spatiotemporal changes of groundwater nitrate concentrations owing to the many sources involved (Galloway *et al.*, 2008; Burow *et al.*, 2010).The different land use types significantly affected the nitrate

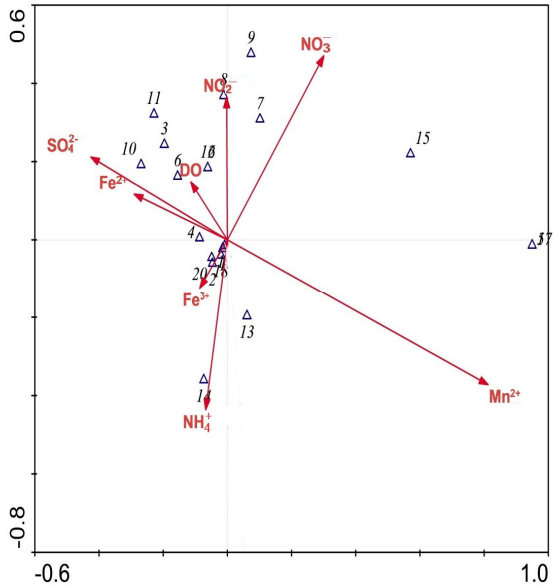

Figure 5. Canonical correspondence analysis (CCA) ordination diagram of DGGE data.

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contamination levels in the groundwater. Therefore, identifying the sources and implementing source control are key issues in mitigating groundwater nitrate pollution.

Composition of Indigenous Microorganism

The DGGE fingerprint patterns showed the composition of the bacterial community in the nitrate contaminated groundwater. Because the very faint bands or the low number of bacterial were not successfully re-amplified or sequenced, the sequenced bands occupied significant proportions in the nitrated groundwater in this area. The strains included *Hyphomicrobium denitrifican* sp., *Halanaerobium praevalens* sp., *Desulfotomaculum reducens* sp., *Nitrosospira multiformis* sp. *Sulfurovum* sp., and *Thiobacillus denitrificans* sp., which were detected among the predominant 16S rDNA gene-based DGGE bands in the groundwater samples of this area.

Denitrification was the most significant attenuation process of nitrate-polluted groundwater, involving the reduction of nitrate via a chain of microbial reduction reactions to nitrogen gas. Among the denitrifying microorganisms, autotrophic bacteria can reduce nitrate to nitrogen gas while oxidizing elemental sulfur or reduced sulfur compounds (S^{2-}, $S_2O_3^{2-}$, SO_3^{2-}) to sulfate, therefore, the denitrifier and sulfate reducing bacteria simultaneously existed in the community. In addition, the metal ions (Fe^{2+}, Mn^{2+}) in the groundwater also could act as the main electron donors. Based on the above results, this area has a great number of denitrifying bacteria, and has the potential to use bioremediation methods to remedy the nitrate contaminated groundwater.

Relationship with Environmental Factors

Depending on all above studies, we had a better known to microbial community structure in the groundwater of Hun River alluvial proluvial fan and their relationship with hydrochemistry factors. Commonly, autotrophic denitrifiers are known as those bacteria able to couple the oxidation of inorganic compounds, such as sulfide, iron, molecular hydrogen, uranium and other metals, to the reduction of nitrate. It is noteworthy that the microbial diversity of denitrifiers in natural environments is not fully understood (Torrentó *et al*., 2011). Our research showed that the composition and distribution of microbial community had a relationship with the concentrations of Mn^{2+}, Fe^{2+}, NO_3^-, NH_4^+, SO_4^{2-} and DO. The most significant factors affecting the microbial community were Mn^{2+} and NO_3^-, the next were Fe^{2+} and SO_4^{2-}. By means of controlling the concentration of these key electron donors, the microbial community structure could be optimized to strengthen the natural attenuation of nitrate or nitrite in the groundwater of this area, which had an important meaning and a reference to other nitrate contaminated fields. The results of the present research suggested that combining genetic fingerprinting of the microbial community with CCA analysis was a convenient and useful method that reflected the environmental conditions in the contaminated sites. As the method continues to be refined, it is expected to play an increasingly important role in eco-environmental hydrogeology.

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رابطه های ترکیب میکروبی و عوامل هیدرو شیمیایی در آبهای زیر زمینی آلوده به نیترات در دشت آبرفتی و دامنه ای رودخانه هون در چین

ا. ز. ژو، ی. ل. ژانگ، ج. ی. دنگ، و ز. س. سو

چکیده

هدف پژوهش حاضر بررسی تنوع جوامع میکروبی و روابط ترکیب میکروبی این جوامع با عوامل هیدرو شیمیایی در آبهای زیر زمینی آلوده به نیترات در دشت آبرفتی رودخانه هون بود. در این بررسی، از روش واکنش زنجیره ای پلیمراز (PCR-denaturing) و الکتروفورز تدریجی ژل(DGGE) شناسایی ژن همراه با تجزیه به روش (canonical correspondence analysis CCA) استفاده شد. واحد های عملیاتی رده بندی (OTU) درهمه نمونه های نقاط مطالعه شده درجه معینی از هتروپلاسمی داشتند و ۷۵٪ از واحد های عملیاتی رده بندی درکمتر از نیمی از نقاط نمونه برداری شده حضور داشتند. تجزیه خوشه ای میانگین غیر وزنی متوسط گروه زوجی (un-weighted pair group mean average (UPGMA نشان داد که شباهت جامعه میکروبی در همه محل های نمونه برداری نسبتا زیاد نبود (۰/۸-۰/۶). نتایج گواه میداد که توزیع جامعه میکروبی با مقدار نیترات به طور مثبتی همبستگی داشت. در آب های زیر زمینی آلوده به نیترات باکتری های غالب شامل بود بر *Hyphomicrobium denitrifican* sp.، *Halanaerobium praevalens* sp، *Desulfotomaculum reducens* sp و *Nitrosospira multiformis* sp که در میان آن ها *Nitrosospira multiformis* sp و *Sulfurovum* sp، در همه نقاط وجود داشتند. نیز، نتایج تجزیه (CCA) حاکی از آن بود که یون Mn^{2+} و NO_3^- موثر ترین عوامل هیدرو شیمیایی در تنظیم ترکیب میکروبی آب های زیر زمینی آلوده به نیترات در ابن ناحیه بودند و یونهای Fe^{2+} و SO_4^{2-} بعد از آنها قرار داشتند. این نتایج مرجعی برای بهسازی زیستی (زیست پالایی)آب های زیرزمینی آلوده به نیترات در دشت آبرفتی رود خانه هون در چین فراهم می کنند.

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