


Article

Spatial Pattern of Bacterial Community Diversity Formed in Different Groundwater Field Corresponding to Electron Donors and Acceptors Distributions at a Petroleum-Contaminated Site

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Received: 27 March 2018; Accepted: 22 June 2018; Published: 25 June 2018



Abstract: The benefits of an electron-transfer mechanism for petroleum biodegrading have been widely acknowledged, but few have studied the spatial pattern of microbial community diversity in groundwater fields, and few discuss the bacterial community's diversity in relation to electron donors-acceptors distribution, which is largely determined by groundwater flow. Eleven samples in different groundwater fields are collected at a petroleum-contaminated site, and the microbial communities are investigated using 16S rRNA gene sequences with multivariate statistics. These are mainly linked to the chemical composition analysis of electron donor indexes COD, BTEX and electron acceptor indexes DO, NO₃[−], Fe²⁺, Mn²⁺, and SO₄^{2−}, HCO₃[−]. The spatial pattern of the bacterial community's diversity is characterized and the effect of the electron redox reaction on bacterial community formation in different groundwater field zones is elucidated. It is found that a considerable percentage (>65%) of the bacterial communities related to petroleum degrading suggest that petroleum biodegrading is occurring in groundwater. The communities are subject to the redox reaction in different groundwater field zones: The side plume zone and the upstream of the source zone are under aerobic redox or denitrification redox, and the corresponding bacteria are *Rhodoferrax*, *Novosphingobium*, *Hydrogenophaga*, and *Comamonas*; the source zone and downstream of the source zone are under Fe³⁺, Mn⁴⁺, and SO₄^{2−} reduction redox, and the corresponding bacteria are *Rhodoferrax*, *Treponema*, *Desulfosporosinus*, *Hydrogenophaga*, and *Acidovorax*. These results imply that groundwater flow plays a definitive role in the bacterial community's diversity spatial pattern formation by influencing the distribution of electron donor and acceptor.

Keywords: groundwater; bacterial community diversity; petroleum contamination; electron acceptor; electron donor; groundwater flow path

1. Introduction

Due to its toxicity to humans and other organisms, there is much concern about petroleum-contaminated groundwater [1,2]. Various methods, including pump-and-treat (PandT) and other in situ technologies have been used to remediate petroleum-contaminated groundwater [3]. As an environmentally sound and cost-effective technology in various applications, in situ

bioremediation, especially natural attenuation, has gained considerable attention. During bioremediation, petroleum degrading microorganisms can mineralize petroleum components as their carbon source and electron donors, electron acceptors redox reaction occurs and the contaminants are removed [4]. Bacterial community diversity and electron-transfer research are essential for bioremediation [5,6].

Research on the diversity of bacterial communities has been carried out at petroleum-contaminated sites since the 1970s [7], and the amounts have grown rapidly as the 16S rRNA gene sequences mature and become universal; most were in soils and sediments [8–12]. A few researchers focusing on groundwater such as Anne Fahy and Zhaoxian Zheng, investigated the relationships between the bacterial community structures and the groundwater geochemistry respectively [13,14], and found that hydrocarbon metabolism would vary the diversity of the bacterial communities. Ai-xia Zhou researched the responses of microbial communities to seasonal fluctuations in groundwater level and found that groundwater-table fluctuations would affect the distribution, transport, and biodegradation of the contaminants [15]. Petroleum compounds can be transported from the source area in groundwater, with the result that the petroleum concentrations, redox conditions, biogeochemical processes, and bacterial communities would vary along the groundwater flow path [16]. This view can be supported by other related research, such as from Karolin Tischer, Etienne Yergeau and C.E. Main who have reported that contaminant concentrations have a significant influence on microbial communities in various environmental mediums [8,17,18]. During petroleum biodegradation, electron donors and acceptors dissolved in the groundwater are consumed. Generally speaking, electron acceptors are usually consumed in the following order: O_2 , NO_3^- , Mn^{4+} , Fe^{3+} , SO_4^{2-} , and HCO_3^- (or CO_2), and these electron acceptors, other than HCO_3^- (or CO_2), are transported in groundwater from uncontaminated groundwater upstream under little vertical recharge conditions. The electron acceptors would be used according to their redox potential and the concentrations of electron acceptors often vary with the groundwater flow path in contaminated sites [19]. Research has shown that certain electron acceptors affect bacterial communities [20,21]. There is enough dissolved oxygen in the edge of contamination plume, and the bacteria are mainly aerobic, while in the source area the bacteria would mainly become methanogens and sulfate-reducing bacteria since the other electron acceptors were already exhausted [22]. There is not much doubt that redox zonation and microbial changes along the path of groundwater plumes are present.

However, for a petroleum-contaminated site bioremediation, microorganisms have cooperative and competitive relationships, not only along a groundwater path. To develop an effective remediation scheme, information about the abundance of petroleum degradation microorganisms from the overall bacterial community diversity is required. The spatial pattern of the diversity of the bacterial community in different groundwater fields of the entire contaminated groundwater area should be well described, and a particularly detailed response regarding the relationship between the bacterial community and electron-transfer will provide field case support for microbial functional gene identification.

Eleven groundwater samples from different places along and beside the groundwater flow path in a petroleum-contaminated aquifer were collected. Then high throughput sequencing of 16S rRNA genes was used to investigate the diversity of the bacterial communities in the samples. The relationships between the bacterial communities and the electron acceptors and donors in the different groundwater fields were assessed. The reasons for a different redox zonation are expected to be gained based on water geochemistry (electron acceptors and available hydrocarbons) and phylogenetic types of microorganisms in the groundwater.

2. Materials and Methods

2.1. Site Description and Sampling Procedure

The contaminated site was located in the northern part of the North China Plain, which was formerly a chemical plant that was contaminated when petroleum leaked from a storage tank more than forty years ago. While the pollution had been removed from the surface, the groundwater and subsurface soils were still seriously contaminated. It reported that the main contaminants were monocyclic aromatics and aliphatic hydrocarbon. More than 11 wells were drilled in the site to survey and monitor the groundwater contamination and then remediate it. The samples were collected before the remediation. The aquifer at the site was mainly composed of sandy gravel and sand, and there was no clay layer to prevent contamination of the vadose zone. Previous studies reported that the contaminated aquifer was unconfined and the depth to the groundwater table was approximately 25 m. The groundwater naturally flowed from northwest to southeast, regionally. Since the site was located in the urban area where there was usage of groundwater, the flow direction varied slightly with time. The flow was from west to east on the sampling days in the site. An area of 400 m² with 11 wells around the petroleum leak was established for the purposes of this study (Figure 1).

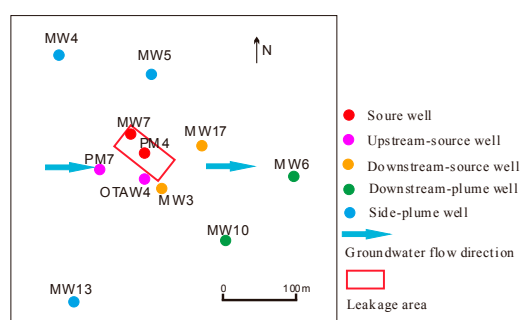


Figure 1. Diagram showing the petroleum-contaminated area, the monitoring wells, and the groundwater flow direction.

Information about the water temperature (t), pH, electrical conductivity (EC), dissolved oxygen (DO), and oxidation-reduction potential (ORP) in each well was recorded before collecting the groundwater samples. The groundwater samples were considered to be representative when the values of T, pH, EC, DO, and ORP in three successive samples were within ± 1 °C, ± 0.2 , $\pm 3\%$, $\pm 10\%$ or ± 0.2 mg/L, and ± 20 mV, respectively. The groundwater samples were collected by sterile bailers. When sampling, groundwater 5 L was collected into a sterilized 5 L plastic bucket and stored on ice in an incubator. The water samples were transported to the laboratory, and the bacteria were collected into 5 PTFE filter membranes with a pore size of 0.22 μm by air pump filtration in one day. The filter membranes were stored at -80 °C until DNA extraction. Other portions of the samples were collected into 500 mL plastic bottles and 40 mL amber glass bottles for inorganic and organic analyses, respectively.

The sampling wells were divided into five groups by the groundwater flow direction and the location of the contamination source. Since the flow direction varied a little with time, the concentrations of contaminants were also considered during the grouping. The upstream-source group included samples PM7 and OTAW4, the source group included MW7 and PM4, the downstream-source group included MW3 and MW17, the downstream-plume group included samples MW6 and MW10, and the side-plume group included samples MW4, MW5, and MW13.

2.2. Chemical Analyses

Considering that monocyclic aromatics are related to their toxicity and relatively high solubility [23], and the chemical oxygen demand (COD) is always used to quantify the amount

of organics in petroleum [24], the concentrations of monocyclic aromatics and COD were monitored as the contamination indexes during the study.

The concentrations of monocyclic aromatics, such as toluene, ethylbenzene, m-xylene, p-xylene, and o-xylene, were determined as outlined in US EPA Method 8260 [25]. Concentrations of other variables, namely chemical oxygen demand (COD), total dissolved solids (TDS), pH, Ca^{2+} , Mg^{2+} , Na^+ , K^+ , NO_3^- , SO_4^{2-} , HCO_3^- , Cl^- , Fe^{2+} , Mn^{2+} , and CO_2 , in the groundwater were measured following the Standard Methods [26].

2.3. DNA Extraction, PCR Amplification, Library Construction and Sequencing

DNA were extracted from each sample using an EZNA™ Soil DNA Kit (OMEGA bio-tek, Norcross, GA, USA) following the manufacturer's protocol. The V4–V5 region of bacterial 16S-rRNA genes was amplified using the universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) [27]. The PCR analysis was carried out in the following order: Initial denaturation at 98 °C for 2 min, 30 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Libraries were sequenced by a sequencing platform (HiSeq 2500) at Personalbio-Shanghai, Shanghai, China.

2.4. Bioinformatics Analysis

Raw sequences were filtered and then high-quality reads were assigned to operational taxonomic units (OTUs) [28]. Then the OTUs were subsampled to the minimum reads. Various alpha-diversity indexes (observed species [Sobs], the Chao estimate, abundance-based coverage estimator [ACE], the Shannon and Simpson diversity indexes) were used to evaluate the species information.

To determine the influence of the petroleum on the bacteria, samples were divided into three groups according to the degree of COD contamination. The samples with COD concentrations less than 10 mg/L, between 10 mg/L and 100 mg/L, and greater than 100 mg/L were classified as having low contamination, medium contamination, and high contamination, respectively. The authors then used Venn diagrams to examine the bacterial communities in the groups by the contamination level and by the groundwater flow areas.

The authors carried out principal coordinate analysis (PCoA) of the microbial communities using unweighted unifrac with full trees at the genus level. The authors used redundancy analysis (RDA) to determine which environmental variables were associated with changes in the structures of the bacterial community. The environmental variables were divided into two groups. One group contained the contaminant compounds, organic index (COD), and the electron acceptors, such as DO, NO_3^- , SO_4^{2-} and HCO_3^- , or metabolic by-products, Mn^{2+} and Fe^{2+} , involved in microbial degradation. The other group contained the pH, TDS, and the major ions in groundwater, such as Ca^{2+} , Mg^{2+} , Na^+ , K^+ , NO_3^- , SO_4^{2-} , HCO_3^- , and Cl^- .

The bioinformatics analyses were carried out in the cloud platform of majorbio (<http://www.i-sanger.com>) according to the programs as the reference mentioned [29].

3. Results

3.1. The Distribution of Electron Acceptors-Donors and Other Chemical Parameters

The concentrations of electron acceptors and donors and other chemical parameters in the groundwater are shown in Table 1. The concentrations of the contaminants, i.e., electron donors and COD, show that the groundwater was seriously contaminated by petroleum and that the contamination varied in the different areas. The contamination was highest in the source area and decreased (in order) in the downstream-source, downstream-plume, upstream-source, and side-plume areas. Apart from the side-plume wells, the DO and NO_3^- concentrations were less than 2 mg/L and 12 mg/L, respectively. The concentrations of HCO_3^- , K^+ , Na^+ , Ca^{2+} , Mg^{2+} , Cl^- , TDS, and CO_2 were lower in the upstream-source wells.

Table 1. Hydrochemical parameters and the contaminant concentrations.

Location		Side-Plume			Upstream-Source		Source	Downstream-Source		Downstream-Plume		
Well		MW5	MW4	MW13	PM7	OTAW4	MW7	PM4	MW3	MW17	MW6	MW10
Electron donors ($\mu\text{g}\cdot\text{L}^{-1}$)	toluene	97	4	6	315	108	11,211	20,610	723	11,680	7689	287
	ethylbenzene	11.1	6.9	0.1	51.7	20	4316	583	832.9	2078	668	971.5
	m(p)-xylene	22.21	4.81	1.24	58.3	25	3001	3678	579.17	1636.5	1115	759.8
	o-xylene	22.22	2.13	0.66	56.67	8.3	3001	3680	320.2	1636.5	225	89.45
Electron acceptors ($\text{mg}\cdot\text{L}^{-1}$)	DO	2.38	2.19	1.95	0.87	0.76	1.29	0.84	1.3	0.85	1.97	1.67
	NO_3^-	16.77	68.96	4.6	1.78	<0.20	1.75	1.76	1.76	1.75	11.5	7.73
	SO_4^{2-}	107.2	277.6	39.67	38.15	69.22	83.79	66.7	21.54	16.68	163.3	98.7
	HCO_3^-	791.6	780.1	889.1	316.7	494.5	648.5	715.5	712.5	831.8	822.1	767.3
metabolic by-products ($\text{mg}\cdot\text{L}^{-1}$)	Fe^{2+}	0.049	0.018	0.372	0.127	0.061	0.489	0.806	0.587	4.643	2.305	1.586
	Mn^{2+}	6.48	0.625	1.953	0.755	0.781	2.356	2.5	1.473	2.589	3.518	2.862
Other ion ($\text{mg}\cdot\text{L}^{-1}$)	K^+	3.14	3.35	2.49	1.58	1.91	2.2	1.72	1.43	2.17	2.57	1.01
	Na^+	179.3	135.9	149.4	67.96	135.8	133.6	137.8	145.6	165.4	124.1	152.4
	Ca^{2+}	282.9	255.9	141.2	61.56	99.63	151.4	162.3	108.9	139.4	209.4	172.8
	Mg^{2+}	121.4	93.74	61.81	21.59	42.24	62.44	59.96	41.58	61.32	81.87	60.19
	Cl^-	583.6	214.6	134.5	61.32	162.7	198.6	195.1	141.8	191.6	182.9	193.4
	TDS	1675	1439	975.3	412.3	758.8	958.1	983.1	818.9	994.3	1177	1064
COD ($\text{mg}\cdot\text{L}^{-1}$)		4.37	1.65	4.54	6.19	56.23	162.4	268.7	56.23	337.4	40.63	31.24
pH		6.94	7.26	7.22	7.59	7.66	7.15	7.02	7.22	6.94	7.15	7.28

3.2. Alpha-Diversity Indexes

Alpha-diversity indexes of the bacterial communities in the 11 samples are shown in Table 2. The good coverage index (>0.995) showed that the obtained reads in the study were representative. The Sobs, Shannon, ACE, and Chao indexes were higher, while the Simpson index was lower, in the samples from the side-plume than in the other samples.

Table 2. Alpha-diversity indexes.

Location	Sample	Diversity Indexes					
		Sobs	Shannon	Simpson	Ace	Chao	Coverage
Side-plume	MW5	383	3.73	0.07	428.3	443.86	0.995
	MW4	324	3.59	0.08	356.17	358.89	0.996
	MW13	249	2.82	0.12	317.9	314.27	0.995
Upstream-source	PM7	211	2.89	0.12	260.23	262.21	0.996
	OTAW4	191	2.76	0.15	240.13	226.25	0.997
Source	MW7	197	2.79	0.12	246.43	238.44	0.996
	PM4	186	2.7	0.2	341.12	294.48	0.995
Downstream-source	MW3	190	2.61	0.17	287.61	263.75	0.995
	MW17	160	2.18	0.2	204.92	196.96	0.997
Downstream-plume	MW6	215	3.44	0.07	266.41	291.56	0.996
	MW10	204	3.05	0.09	256.63	255.11	0.996

3.3. Community Composition

The compositions of the bacterial communities in the 11 samples at the genus level are shown in Figure 2.

Sequences representing *Dechloromonas* were the most abundant bacterium and accounted for 23% of all bacterial sequences in MW7. *Dechloromonas* was also detected and accounted for between 3% and 9% of all the bacteria, in PM4 and in the samples collected from the downstream-source and downstream-plume areas.

Sequences representing *Acidovorax* were most the abundant, and accounted for 43% of all bacteria, in PM4. They were also present at abundances greater than 10% in MW7, and in samples from the downstream-source (MW17 and MW3) and downstream-plume (MW6) areas. These sequences were also present at lower abundances in other samples.

Sequences representing *Hydrogenophaga* were most the abundant in OTAW4, MW13, MW17, and MW3, where they accounted for 33%, 45%, 38%, and 37%, respectively. They were also present at abundances of between 3% and 20% in all other samples, except those from the contamination source area (samples MW7 and PM4).

Sequences representing *Comamonas*, present in all samples, were the most abundant in samples PM7 and MW4, where they accounted for 23% and 19% of all bacteria, respectively. They were most abundant in the upstream source area, and then decreased (in rank order) in the side-plume, downstream-source, downstream-plume, and were least abundant in the contaminant source areas.

Sequences representing *Rhodoferrax* were the most abundant in MW5, MW6, and MW10, where it accounted for 23%, 18%, and 23% of total bacteria, respectively. Except for sample PM4, *Rhodoferrax* accounted for more than 10% of all bacteria in the samples.

Sequences representing other bacteria were present at higher abundances (>5%) in certain samples. *Pseudomonas* was in all the samples, but only had abundances of more than 10% in samples from the source area (MW7 and PM4) and in MW10, for example. *Treponema* was detected in all samples and, apart from MW7 and MW10 where it had abundances of 7% and 10%, respectively, its abundances were less than 5%. Present in all samples, *Novosphingobium* was more abundant in the upstream-source area samples (OTAW4 (12%) and PM7 (8%)) than in other areas, where its abundances were less than 5%. *Pseudoxanthomonas* was present in samples PM4 and MW7 from the source area at abundances of 6% and 9%, respectively, and was either present at abundances of less than 1% in, or was absent from, the plume area. *Zavarzinia* was most abundant in MW4 (12%) and MW6 (8%) and was present in other samples at abundances of less than 1%. *Sulfuritalea* was detected in all samples. Apart from MW5, where its abundance was 13%, its abundances were less than 2%. *Sulfuricurvum* had abundances of 7% in MW5 and MW10, and of about 1% in MW3 and MW6. *Desulfosporosinus* was only present in MW6 and MW10 at abundances of 2% and 8%, respectively. *Nitrospira* was found in MW5 and MW4 only at abundances of 5% and 1%, respectively. *Norank_p__Omnitrophica* was only found in MW5 at an abundance of 5%.

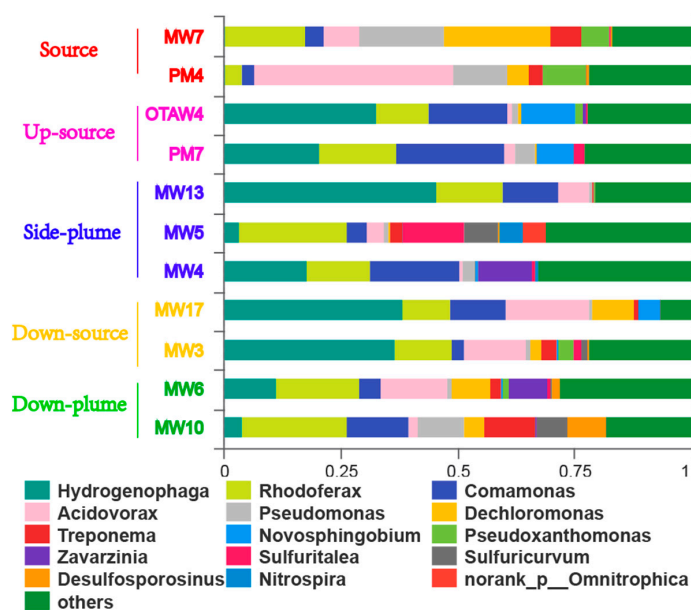


Figure 2. Bar chart of community abundances (greater than 5%) at the genus level.

3.4. Relationships between Bacterial Communities among Samples

Venn diagrams showed the number of species that were unique to, or shared between, the different groups. The species are presented according to the different degrees of petroleum contamination in Figure 3a. The 3 contamination classes shared 214 species, and the group with low contamination

had 191 unique species. This figure demonstrates the reduced bacterial diversity in petroleum-contaminated wells. The species found in the different parts of the groundwater flow field are shown in Figure 3b. The different parts shared 98 species, and the groundwater from the side-plume area had the greatest number of unique species.

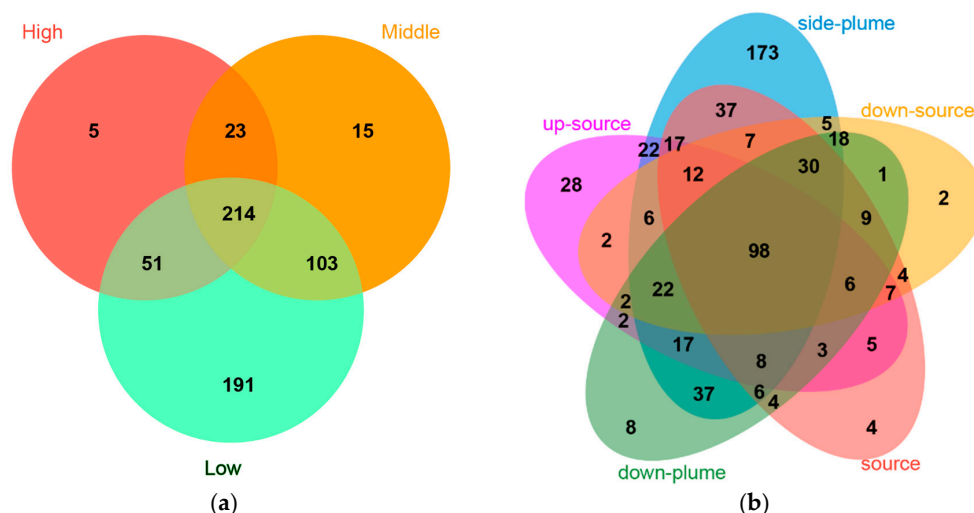


Figure 3. Venn diagrams showing the bacteria by (a) the degree of contamination and (b) locations.

The results of the PCoA are shown in Figure 4. The samples from the same groundwater flow area were grouped together. The side-plume group was grouped to the left of the graph along the first principal component (PC1), while the other samples were grouped to the right of the graph. The samples from the upstream-source, downstream-plume, downstream-source, and source areas were grouped along the second principal component (PC2).

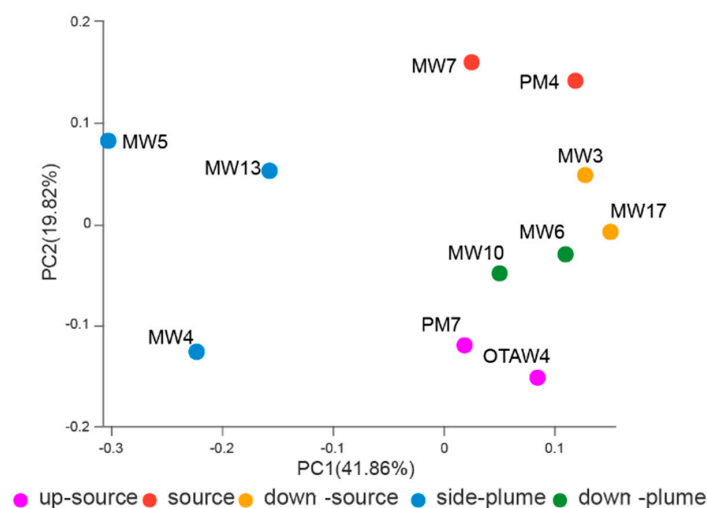


Figure 4. The PCoA plot of samples at the genus level.

The RDA plot (Figure 5) indicated that samples PM4 and MW7 from the contamination source area and sample MW6 from the downstream-plume were associated with high concentrations of COD, toluene, xylene, and ethylbenzene. Samples MW5 and MW10 were associated with high SO_4^{2-} and DO, samples MW4 and PM7 were associated with high NO_3^- and DO, sample MW13 was associated with low Mn^{2+} , and samples MW3 and MW17 were associated with high Fe^{2+} and low SO_4^{2-} . The upstream-source samples were associated with high pH values.

Most of the genera were clustering in the center of the plot, which indicates that these bacteria were present in all samples at similar abundances. The relative abundance of *Acidovorax* increased as the COD increased. *Pseudoxanthomonas* was positively correlated with toluene and xylene, and *Novosphingobium* and *Comamonas* were negatively correlated with toluene and xylene. *Dechloromonas* and *Pseudomonas* were positively correlated with ethylbenzene. *Hydrogenophaga* was negatively correlated with bivalent manganese (Mn^{2+}). *Rhodoferrax* was negatively correlated with Fe^{2+} and positively correlated with DO.

The index of the organic content, COD, was positively correlated with various contaminants (toluene, xylene, and ethylbenzene) and HCO_3^- , Fe^{2+} , and Mn^{2+} , and was negatively correlated with SO_4^{2-} , DO, and NO_3^- . The pH was negatively correlated with TDS, Mg^{2+} , Ca^{2+} , and Cl^- .

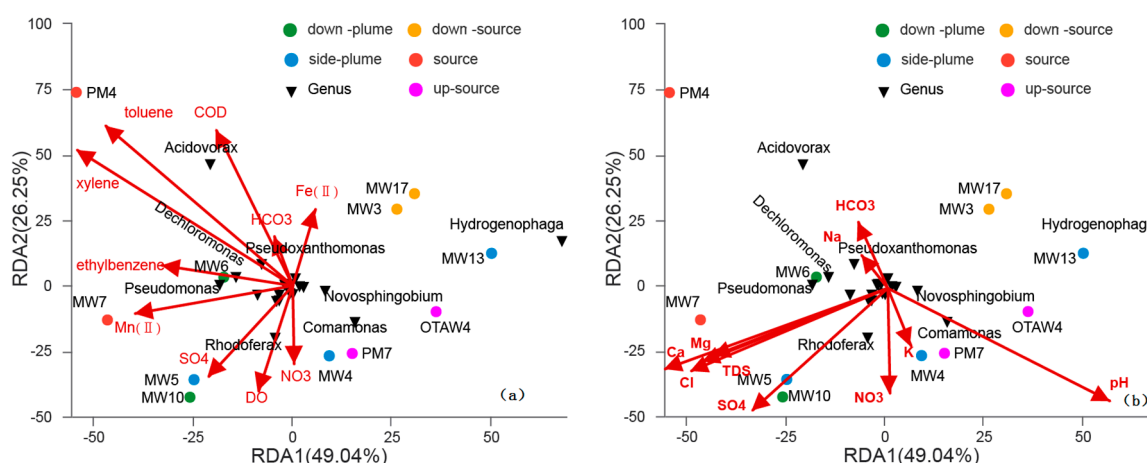


Figure 5. Redundancy analysis (RDA) of the relationship between the groundwater parameters and the relative abundance of the bacterial genus of the collected samples. The contamination indexes and the electron acceptors are mainly shown in (a); while the major ions, TDS, and pH are mainly shown in (b).

4. Discussion

4.1. Variations in Bacterial Communities with Electron Donor Concentrations

Analysis of the community diversity of all the groundwater samples showed that the bacterial communities of samples within the same groundwater flow area were similar. Most of the bacteria that were present at abundances of more than 5% in this contaminated aquifer were related to the degradation of hydrocarbons, especially aromatic hydrocarbons. The bacterial communities varied in different areas.

Hydrogenophaga was not present in the source zone (MW7 and PM4). *Hydrogenophaga* can metabolize various organic compounds, such as polycyclic aromatics and toluic acid, but not toluene and xylene [30]. Researches showed that high concentrations of toluene and xylene may have negatively impact it [31], *Hydrogenophaga* tends to be more abundant where toluene and xylene concentrations are low and COD concentrations are high. *Comamonas* can also degrade polycyclic aromatic hydrocarbons [32], but its abundances were lowest in the source area (samples MW7 and PM4). This bacterium might be harmed by high concentrations of hydrocarbons. While *Rhodoferrax* can degrade benzene [33], it was least abundant in PM4 where the toluene contamination was greatest, which might indicate that this bacterium is sensitive to toluene. *Rhodoferrax* was correlated with increases in DO in the RDA plot, which suggests that its growth may also be limited by oxygen. *Acidovorax*, *Pseudomonas*, *Dechloromonas*, and *Pseudoxanthomonas*, also capable of degrading hydrocarbons, were abundant in the highly contaminated groundwater in the source zone. *Acidovorax* can use various PAHs [34]. The species of *Pseudomonas* in the study site was *Pseudomonas mendocina*, which can degrade toluene [35]. The *Dechloromonas* in the aquifer shared a 99% sequence similarity with *Dechloromonas hortensis*

and *Dechloromonas denitrificans*, which can use ClO_4^- , ClO_3^- , NO_3^- , and O_2 as electron acceptors to oxidize organic compounds, such as aromatic hydrocarbons [36,37]. *Pseudoxanthomonas* can produce biosurfactants and can be used to degrade BTEX [38,39]. These four bacteria, *Acidovorax*, *Pseudomonas*, *Dechloromonas* and *Pseudoxanthomonas*, may be more tolerant to petroleum than the other bacteria, as shown in the RDA analysis. *Novosphingobium* can also degrade aromatic hydrocarbons [40,41] and was more abundant in upstream-source zone samples (OTAW4 and PM7) than in the other areas. *Treponema*, previously discovered in hydrocarbon-contaminated sediments, can degrade hydrocarbons [8,42] and was present at relatively high abundances in MW7 and MW10. *Zavarzinia*, which can degrade benzene in aerobic conditions [43], was mainly found in samples MW4 and MW6 from the plume area, where the contents of dissolved oxygen were relatively high. Apart from sample MW5 from the side-plume, these 10 hydrocarbon degrading bacteria mentioned above accounted for more than 65% of all bacteria in all the samples.

The high abundance of degradation bacteria in the groundwater samples, combined with electron acceptors-donors, indicates that natural attenuation was occurring at this site. Venn diagrams (Figure 3) and the α -diversity indexes (Table 2) showed that a considerable number of species were sensitive to petroleum organics. The values of the Sobs, Chao, ACE, and Shannon indexes were higher, while the Simpson index was lower, in the samples from the side-plume than in the other samples. The Sobs, Chao, and ACE indexes are used to reflect the richness of species, and the Shannon and Simpson indexes reflect both the richness and the evenness of the species in samples [44]. The Shannon index has been reported to be more sensitive to changes in richness, while the Simpson index is more sensitive to the evenness [45]. Therefore, it can be said that, consistent with other studies [11,46], contamination can result in a decline of diversity and increase of abundances of dominant microorganisms. The RDA plots indicated that, of all the environmental factors, the toluene, xylene, and COD had the most effect on the bacterial communities.

4.2. The Influence of Electron Acceptors on Bacterial Communities

The electron acceptors were being consumed as the petroleum was being degraded. The electron acceptors were consumed in a certain order, with O_2 consumed first, followed by NO_3^- , Mn^{4+} , Fe^{3+} , SO_4^{2-} , and HCO_3^- , during aerobic reaction, denitrification, Mn^{4+} reduction, Fe^{3+} reduction, SO_4^{2-} reduction, and methane production, respectively [16].

Samples MW3 and MW17 were associated with high Fe^{2+} and low SO_4^{2-} , which indicated that the electron acceptors Fe^{3+} and SO_4^{2-} had been consumed, these two samples probably were in the methanogenesis stage. These two samples had the highest abundances of *Hydrogenophaga*, which is always closely related with methanogenic archaea [47,48]. Other studies have suggested that *Hydrogenophaga* might have catalyzed hydrogen production or perhaps was an oxygen scavenger, and that it created the strictly anoxic conditions essential for the methanogenic archaea [49]. *Hydrogenophaga* can produce and use hydrogen, one of the substrate of methanogenesis, as its energy source, which suggests that there might be cooperative relationships between *Hydrogenophaga* and methanogenic archaea. Sample PM4 was associated with high Fe^{2+} and Mn^{2+} , so either Fe^{3+} , Mn^{4+} , and SO_4^{2-} reduction or methanogenesis might have dominated. However, to the best of the authors' knowledge, the two most abundant bacteria in the sample, *Acidovorax* and *Pseudomonas*, cannot use Fe^{3+} , Mn^{4+} , or SO_4^{2-} as their electron acceptors [50,51], which suggests that methanogenesis might have also dominated in this area. Other studies have shown that *Acidovorax*, a facultative aerobic microorganism that can use carboxylic acids as a carbon source, was closely associated with methanogenic archaea [49]. The sequences that represent syntrophic bacterial species, *Syntrophus*, *Syntrophomonas*, *Syntrophobacter* and *Pelotomaculum*, which usually provide hydrogen and carbon dioxide to methanogens, were found in these samples (See OTU table in Supplementary Materials).

Samples MW7 and MW6 were associated with high Mn^{2+} and SO_4^{2-} , which suggests that these samples were in the Fe^{3+} reduction phase and had the potential to reduce the SO_4^{2-} . *Dechloromonas*, *Rhodoferrax*, and *Pseudomonas mendocina* were the most abundant bacteria in these two samples.

Dechloromonas often use NO_3^- and O_2 as electron acceptors, and are not known to grow in Fe^{3+} or SO_4^{2-} reducing conditions, or through syntrophic interactions with methanogenic bacteria [52]. *Rhodoferrax* can use Fe^{3+} , NO_3^- , and O_2 , but not SO_4^{2-} as electron acceptors [53]. *Pseudomonas mendocina* tested positive for oxidase and NO_3^- reduction (assimilatory), but negative for dissimilatory NO_3^- , Fe^{3+} , and SO_4^{2-} reduction [54]. While these bacteria cannot use SO_4^{2-} as an electron acceptor, *Treponema* [55] and *Desulfosporosinus* [56], found in samples in MW6 and MW7, can reduce SO_4^{2-} .

Samples MW5, MW10, and MW4 were associated with high SO_4^{2-} , DO, and NO_3^- , which indicated that these samples were in the aerobic or denitrification phase. *Rhodoferrax*, as shown in the RDA plot, was related with these samples. The groundwater samples contained some special bacteria. *Sulfuritalea*, was detected in all samples at abundances less than 2%, apart from MW5, which had a *Sulfuritalea* content of 13%. This bacterium was isolated from the water of a freshwater lake. It can oxidize thiosulfate, elemental sulfur, and hydrogen as sole energy sources for autotrophic growth and can use NO_3^- as an electron acceptor [57]. *Sulfuricurvum*, which had relatively high abundances in MW5 and MW10, is a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium [58]. *Desulfosporosinus*, was also abundant in MW10, which suggests that there was strong SO_4^{2-} reduction in MW10. *Nitrospira*, was only present in MW5 (5%) and MW4 (<1%). It is a ubiquitous bacterium that can oxidize the NO_2^- into NO_3^- , which is an aerobic process [59]. The relatively high oxygen contents in the two samples meant that there was enough oxygen for this bacterium to survive. *Norank_p__Omnitrophica* only appeared in MW5. More than 10 OTUs corresponded to this bacterium. The function of this bacterium is not well defined; it was previously identified in an anammox community [60] and might be related to Magnetotactic bacteria [61].

Samples PM7 and OTA4 were associated with high NO_3^- and low Mn^{2+} , which suggests that these samples were in the denitrification phases. The positions of the bacteria *Comamonas*, and *Novosphingobium* on the RDA plot, confirm this view. *Comamonas* can reduce Fe^{3+} , Mn^{4+} , and NO_3^- [62,63], but not SO_4^{2-} , and *Novosphingobium* can reduce NO_3^- [40]. The HCO_3^- contents in PM7 and OTA4 were lower and the pH values were higher than in the other samples. When the pH is higher, CaCO_3 and MgCO_3 precipitate more readily [64], resulting in lower TDS, Mg^{2+} , Ca^{2+} and HCO_3^- concentrations in the water.

Sample MW13 was associated with low Mn^{2+} and closely related with *Hydrogenophaga*, which can oxidize Mn^{2+} [65] also. During this process, O_2 and NO_3^- can be used as electron acceptors [48,66]. This suggests that the O_2 and NO_3^- were adequate and MW13 was in the aerobic reaction or denitrification stage.

5. Conclusions

The bacterial community diversity varied within different groundwater flow fields. Suspected hydrocarbon degrading bacteria accounted for a considerable percentage of these bacterial communities, which indicates that petroleum biodegradation potential was great. The different bacterial communities corresponded to different redox reaction stages in different locations. Generally, the side-plume and upstream-source samples were in the aerobic or denitrification stages, and the corresponding bacteria were *Rhodoferrax*, *Novosphingobium*, *Hydrogenophaga*, and *Comamonas*. Samples from the source and the downstream-source areas were related to Fe^{3+} , Mn^{4+} , and SO_4^{2-} reduction and likely methanogenesis, and the corresponding bacteria were *Rhodoferrax*, *Treponema*, *Desulfosporosinus*, *Hydrogenophaga* and *Acidovorax*. It was proposed that spatial patterns of bacterial communities were determined by groundwater flow for its influence on the distribution of electron donors and acceptors in this petroleum-contaminated aquifer.

Supplementary Materials: Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/10/7/842/s1>. Table S1: OTU table.

Author Contributions: M.Z. and Z.H. Conceived and designed the experiments; M.Z., Z.H., Z.N., P.C., C.G. and P.W. collected the samples and performed the experiments; Z.N. and M.Z. analyzed the data and wrote the paper.

Funding: This research was funded by [National Natural Science Foundation of China] grant number [41402233, 41602261], [Natural Science Foundation of Hebei Province] grant number [D2016504021] and [Institute of Hydrogeology and Environmental Geology, CAGS Research Fund] grant numbers [SK201702, SK201604, YYWF201519, SK201614].

Acknowledgments: We thank Deborah Ballantine, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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