

Article

Study on Natural Attenuation of Groundwater Organic Pollutants by Integrating Microbial Community Dynamics and Isotope Analysis

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Abstract: This study focuses on a typical chemically contaminated site in the southeastern coastal region of China, investigating the natural attenuation mechanisms of benzene and o-toluidine in groundwater through high-throughput sequencing, physicochemical analyses, and stable isotope techniques. The results demonstrate significant heterogeneity in the spatial distribution and degradation processes of pollutants within the contaminated zones (W27, W28, W31). Environmental factors such as HCO_3^- , SO_4^{2-} , and ORP predominantly influence the microbial community structure and functional distribution. Stable isotope data reveal that δD and $\delta^{13}\text{C}$ enrichment effects are most pronounced in the deep layer (W28_40m), indicating active pollutant degradation, while degradation in the deeper layers of W27 and W31 is constrained by anaerobic conditions and reduced microbial activity. The combined analysis of hydrogen and carbon isotopes elucidates the degradation pathways and dynamic processes of pollutants within the contaminated zones, providing quantitative evidence for natural attenuation mechanisms and scientific support for optimizing site remediation strategies.



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Keywords: natural attenuation; microbial community; isotope; barrier technology

1. Introduction

Groundwater contamination is an important environmental problem globally, especially in areas of high industrial activity [1]. Chronic chemical spills and improper waste management lead to the accumulation of organic contaminants in groundwater, posing a significant threat to ecosystems and public health [2]. Organic contaminants, such as benzene and o-toluidine, are particularly hazardous due to their high toxicity, persistence, and mobility [3]. These contaminants can penetrate aquifers and remain in groundwater for long periods, potentially causing serious harm to ecosystems and human health [4,5]. In addition, contaminant transport and degradation exhibit significant spatial and temporal heterogeneity, a process that is influenced by the complex hydrogeochemical conditions of the groundwater system, further increasing the difficulty of management [6,7].

In recent years, many physical, chemical, and biotechnological approaches have been applied to groundwater contamination management, including pump and treat, chemical oxidation, thermal desorption, and bioremediation. However, these technologies are often

costly and cause greater disturbance to the ecosystem, especially in low-permeability formations with limited effectiveness [8,9]. Therefore, as a more sustainable and cost-effective alternative, natural attenuation (NA) is gradually becoming a technique of interest, favored for its low intervention, cost effectiveness, and environmental friendliness. Natural attenuation refers to the degradation or transformation of pollutants through physical, chemical, and biological processes without human intervention. Microbiologically mediated biodegradation is a key process in the removal of organic pollutants. Microorganisms convert pollutants into harmless products through complex metabolic pathways in which environmental factors such as redox potential, dissolved oxygen, and the availability of carbon sources play an important role in the degradation process [10,11]. Although the degradation pathways and key microbial species of single contaminants have been investigated, there is limited research on the natural attenuation mechanisms of co-existing contaminants. At contaminated sites, contaminants are often present in mixtures, and interactions between contaminants may affect the structure and function of microbial communities [12,13]. In addition, the spatiotemporal variation in environmental factors and their effects on pollutant degradation and microbial dynamics remain understudied. Several studies have shown that spatially varying physicochemical parameters (such as retardation) can significantly affect the migration of pollutants in groundwater, complicating pollution management [14]. For example, parameters such as permeability, pH, ion concentration, and changes in the redox environment can affect the spatial distribution and migration of contaminants, with more mobile contaminants being more easily transported in certain groundwater zones [15]. Changes in groundwater flow, temperature, and chemical composition can lead to different degradation rates and mechanisms at different locations within a site [16,17]. The hydrogeochemical conditions of a contaminated site can create environments that enhance or inhibit contaminant degradation. For example, in areas with high ion concentrations, microbial activity may be promoted due to nutrient availability, while in low-conductivity environments, microbial metabolism may be limited, reducing degradation efficiency [18]. In this context, understanding how physicochemical factors such as pH, electrical conductivity (EC), redox potential (ORP), and ion concentration affect contaminant distribution and microbial activity is critical to developing effective natural attenuation strategies [19]. While previous research has advanced our understanding of groundwater contamination and natural attenuation, key mechanisms remain underexplored, particularly regarding the interactions of co-existing contaminants and microbial community responses. Most studies have focused on single contaminants or single-method approaches, which fail to capture the full complexity of these processes. Notably, the influence of horizontal and vertical barriers on contaminant transport and distribution has not been adequately addressed. To bridge these gaps, this study integrates microbial community analysis with stable isotope techniques, providing a more holistic approach to understanding the degradation dynamics of co-existing pollutants and their interaction with environmental factors, often overlooked in traditional studies.

This study focuses on a typical chemically contaminated site in the southeastern coastal area of China, which is characterized by horizontal and vertical barriers designed to effectively control the spread of contaminants. These barriers provide a unique environment for studying natural attenuation mechanisms in groundwater. The main goal of this study is to explore the natural attenuation process of co-existing pollutants such as benzene and o-toluidine by combining high-throughput sequencing, physicochemical analysis, and stable isotope technology. Specific objectives include (1) analyzing hydrogeochemical data, studying the spatial heterogeneity of pollutant migration and microbial activity, and revealing the impact of physicochemical factors on natural attenuation; (2) using high-throughput sequencing and stable isotope technology to gain a deep understanding of

the dynamics of microbial communities and pollutant degradation pathways in complex multi-pollutant environments; and (3) combining environmental factor analysis, microbial ecology, and isotope technology to provide a scientific basis for optimizing the management of similar contaminated sites with barriers and promote the management and remediation of similar sites.

2. Materials and Methods

2.1. Study Area

The study area is located on the site of a former chemical plant on the southeast coast of China, which used to produce a wide range of chemicals, including acetic acid, tetra sulphonyl pyrrolidone, pyrazolone, diacetyl, diene ketone, and butyrolactone. The area is a typical industrial area and has been subjected to industrial activities for a long time, resulting in serious groundwater contamination. Regarding hydrogeological conditions, the lithology of the aquifer in the study area mainly consists of flat fill, sandy chalk, silt, and powdery clay and the water table is high in the area. The horizontal and vertical permeability coefficients of the aquifer range from 2.0×10^{-5} to 3.53×10^{-6} and 5.0×10^{-5} to 7.53×10^{-6} , respectively. The groundwater flow direction is mainly from southwest to northeast, and the migration of contaminants in the groundwater shows significant spatial heterogeneity due to the influence of topography and regional hydrological conditions. The layout of the monitoring wells is shown in Figure 1. The study area is surrounded by a river on the east side, which is an important artificial waterway connecting the Yangtze River and the Grand Canal; industrial areas on the south and west sides; and residential areas adjacent to the north side. To control the spread of pollution, several preventive and control measures have been taken, including the construction of vertical and horizontal barriers. The vertical barriers are made of materials with a permeability coefficient of less than 1×10^{-7} cm/s to a depth of about 40 m and are embedded in an impermeable layer of at least 2 m, while the horizontal barriers are made of compacted clay or soil mixed with bentonite, not less than 46 cm thick, with a permeability coefficient of no more than 10^{-6} cm/s. These measures have played an important role in effectively limiting the spread of pollutants to the surrounding area.

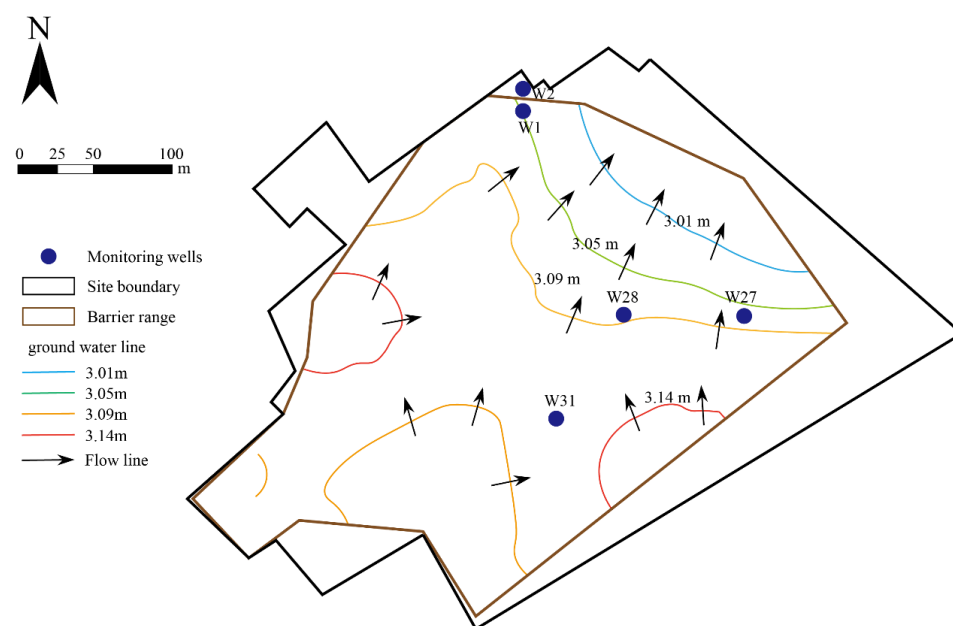


Figure 1. The site and the locations of monitoring wells.

According to the preliminary investigation data, the main pollutants in the study area are chlorinated hydrocarbons and benzene compounds, and benzene and o-toluidine, in particular, are the most dangerous pollutants. The distribution of these pollutants in groundwater shows obvious spatial differences, and the concentration varies greatly in soil layers at different depths. The distribution of pollutants in aquifers at different depths varies considerably due to hydrogeological conditions and the migration characteristics of the pollutants, which further affects the degradation and natural attenuation process of the pollutants.

2.2. Groundwater Sample Collection

Sampling work was conducted in April 2024, using sampling equipment to collect groundwater samples. Before sampling, the groundwater was drawn until the pH value, electrical conductivity (EC), oxidation–reduction potential (ORP), temperature, and dissolved oxygen (DO) measurements reached a stable state. To analyze the spatial differences in the super-risk contaminants and microbial communities in the area, each sampling point was sampled at depths of 6 m, 15 m, and 40 m below the ground surface. Groundwater samples were collected in sealed bottles and transported to the laboratory for further analysis.

2.3. Physicochemical Analysis

This study analyzed two volatile organic pollutants, benzene and o-toluidine, in the groundwater. Both compounds were extracted using liquid–liquid extraction with dichloromethane as the solvent and analyzed by gas chromatography–mass spectrometry (GC-MS). A DB-25 capillary column was used, with a flow rate of 2 mL/min. The oven temperature was initially set to 40 °C for 2 min, followed by a ramp to 100 °C at 5 °C/min and then to 200 °C at 10 °C/min, and held at 200 °C for 8 min. The temperature was then increased to 280 °C at 30 °C/min and held for 3 min. Samples were injected in a 50:1 split mode. Mass spectrometry conditions included a solvent delay of 2 min. The recovery rates, determined through both the blank-spiking and matrix-spiking experiments, were all at least 80% for each compound.

2.4. DNA Extraction, PCR, and High-Throughput Sequencing

In March 2024, 2 L groundwater for microbial community analysis was filtered on the spot using a self-assembled portable filter system (−90 kPa) and microfiltration membranes (0.22 µm) in triplicate. The entire experimental procedure encompassed sample preparation, DNA extraction, PCR amplification, library construction, and high-throughput sequencing, all aimed at ensuring the acquisition of high-quality sequencing data. First, after extracting the genomic DNA, its quality was assessed using 1% agarose gel electrophoresis. Next, PCR amplification was performed using specific primers with barcodes or fusion primers with mismatched bases to target the specified sequencing region. To ensure the accuracy and reliability of subsequent data analysis, amplification was performed under the following two conditions: using the lowest possible cycle number for amplification, and ensuring that all samples were amplified at the same cycle number. Representative samples were randomly selected for pre-testing to ensure that the majority of samples could amplify PCR products of appropriate concentration at the lowest cycle number. PCR amplification was performed using TransGen AP221-02: TransStart Fastpfu DNA Polymerase, and the amplified PCR products were detected by agarose gel electrophoresis with 1% agarose to check the amplified band sizes. The amplified PCR products were detected by 1% agarose gel electrophoresis to determine the size of the amplified bands and purified using an Agen-court AMPure XP Nucleic Acid Purification Kit. Following this, library construction for the second-generation high-throughput sequencing platform was performed, including the ligation of Y-junction adapters, removal of self-ligated adapter fragments, PCR amplifica-

tion to enrich templates, and the generation of single-stranded DNA fragments by sodium hydroxide denaturation. Finally, sequencing was performed on a second-generation high-throughput sequencing platform, where DNA clusters were generated through bridge PCR amplification, and DNA sequences were synthesized using fluorescently labeled dNTPs. Each step of the experimental process was rigorously controlled to ensure data quality, providing a solid and reliable foundation for subsequent microbial community analysis.

3. Results and Discussion

3.1. Hydrogeochemical Variations and Their Impact on Pollutant Distribution in Groundwater

The hydrogeochemical conditions of groundwater are critical in influencing the distribution of contaminants and microbial activity [20]. In March 2024, hydrogeochemical parameters and contaminant concentrations in the study area showed significant spatial variation (Table 1). The pH of groundwater samples ranged from 6.14 to 6.68, generally approaching neutrality, which favors microbial growth and metabolism [21]. However, within the contaminated zone inside the barrier (W31), the pH at the shallow sampling point W31_6m was 6.14, slightly lower than at other locations, likely due to localized acidification from the anaerobic degradation of contaminants. In contrast, sampling points outside the barrier (W2) exhibited higher pH values, with the shallow point W2_6m reaching 6.67, indicating that the barrier effectively restricted contaminant diffusion.

ORP is a key indicator among hydrogeochemical parameters, reflecting the redox state of groundwater, which in turn affects the metabolic activity of microbial communities. In the study area, ORP values ranged from 39 mV to 125 mV, showing significant spatial variation [22]. In uncontaminated areas (such as W1), the ORP value was high (125 mV), indicating that the oxidative environment promotes the activity of aerobic microorganisms and can degrade organic pollutants more efficiently. Taking W1_6m as an example, the ORP was 125 mV and the DO concentration was 2.28 mg/L, indicating that there is sufficient oxygen, which is conducive to aerobic degradation [16]. In contrast, the ORP value in the deep layer of the contaminated area (such as W31_40m) was low (39 mV), indicating that the area is in a reducing environment, which may be conducive to the growth of anaerobic microorganisms and the occurrence of anaerobic degradation [23]. EC is an indicator of the concentration of dissolved ions in groundwater and has an important impact on the metabolism of microbial communities. Areas with higher EC values generally indicate higher ion concentrations, which help support the growth of certain microbial groups. For example, the EC value of W1_6m was 1.84 mS/cm, which is consistent with the higher DO concentration (2.28 mg/L) and the higher degradation rate of pollutants [15]. Higher EC may indicate that there are more dissolved ions in the water, providing more nutrient sources for microorganisms, which is conducive to promoting the degradation of pollutants. However, areas with lower EC values, such as W31_40m (EC of 4.62 mS/cm), indicate that there are fewer dissolved ions here and microbial activity may be limited to a certain extent, which may also affect the degradation efficiency of pollutants [24]. Low-conductivity environments with limited microbial metabolism often mean fewer available ions and nutrients, thereby reducing the degradation rate [22]. The distribution of total organic carbon (TOC) and total nitrogen (TN) in the study area is closely related to microbial activity and pollutant degradation. The data showed that in polluted areas (such as W31_15m), TOC and TN concentrations were high, 578.5 mg/L and 548.5 mg/L, respectively, indicating that these areas have higher concentrations of organic matter and nitrogen sources, providing sufficient nutrients for microorganisms and promoting the degradation of organic pollutants [23]. In contrast, at W2_6m outside the isolation layer, TOC was 55.00 mg/L and TN was 48.58 mg/L, showing less pollution impact [15].

Table 1. Groundwater hydrogeochemical parameters and characteristic contaminants in March 2024.

		T	pH	DO	ORP	EC	K ⁺	Ca ²⁺	Na ⁺	Mg ²⁺	HCO ₃ [−]	Cl [−]	SO ₄ ^{2−}	Mn	Fe	TOC	TN	Benzene	o-toluidine
Number	Depth	°C	/	mg/L	mV	mS/cm	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
W1	6 m	18.3	6.58	2.28	125	1.84	26.20	164.8	122.7	33.33	487.5	93.13	350.6	17.46	3.091	36.42	23.87	0.089	0.014
	15 m	18.5	6.46	2.25	125	2.57	12.46	431.9	32.81	17.26	361.3	481.8	63.64	23.03	5.192	77.50	73.00	0.089	0.011
	40 m	18.6	6.44	2.65	125	3.63	16.27	377.8	100.9	165.4	521.9	755.7	32.26	33.05	16.34	35.30	26.41	0.089	0.008
W2	6 m	17.4	6.67	1.63	109	1.86	21.61	381.7	43.68	39.69	430.1	188.7	230.0	60.75	10.29	55.00	48.58	0.089	0.088
	15 m	18.7	6.68	2.45	91	1.76	11.70	294.0	16.25	4.720	177.8	417.5	57.00	5.25	4.771	94.50	90.50	0.089	0.089
	40 m	18.6	6.48	1.58	61	4.30	19.32	933.4	123.8	138.0	470.3	1153	80.74	35.59	30.54	45.46	41.23	0.089	0.088
W27	6 m	16.3	6.35	1.62	57	3.67	15.50	415.5	294.9	59.03	625.2	231.9	963.4	90.26	9.009	70.50	60.00	0.092	2.168
	15 m	15.8	6.47	2.15	76	3.01	4.11	36.55	155.4	16.18	1141	199.9	91.71	8.285	2.672	134.5	122.0	0.113	42.97
	40 m	16.1	6.52	2.32	42	3.22	13.22	55.89	224.2	24.89	1135	251.7	175.6	17.46	2.254	56.50	50.00	0.118	34.45
W28	6 m	16.8	6.38	1.48	47	3.01	12.46	541.3	109.9	90.37	1026	178.9	1017	181.9	30.09	149.5	134.0	0.100	0.804
	15 m	16.4	6.4	1.86	57	3.28	13.22	39.87	116.3	23.23	1130	83.70	372.2	4.050	5.192	82.00	72.00	0.126	21.89
	40 m	16.3	6.44	1.36	86	2.81	19.32	75.17	127.1	43.22	986.5	129.1	234.5	60.76	10.72	65.00	53.00	0.091	0.198
W31	6 m	16.5	6.14	0.86	49	3.51	10.18	957.7	66.84	77.63	957.9	139.5	1180	78.05	10.72	320.0	305.0	0.090	0.094
	15 m	16.6	6.32	0.78	46	6.46	10.18	32.12	325.4	50.42	1594	686.8	228.6	12.55	14.59	578.5	548.5	0.102	1.640
	40 m	16.6	6.40	1.27	39	4.62	34.63	63.96	160.5	47.40	1451	427.9	218.9	33.69	6.037	198.0	174.5	0.118	0.1167

As shown in Figure 2, the spatial distribution of characteristic pollutants shows significant differences between polluted and unpolluted areas. Benzene concentrations ranged from 0.089 to 0.126 mg/L, while o-toluidine concentrations ranged from 0.014 to 42.97 mg/L. Contaminant concentrations were significantly higher in contaminated areas within the barrier (W27, W28, W31) compared to uncontaminated areas (W1, W2). For example, at W27_15m, o-toluidine concentrations reached 42.97 mg/L, several times higher than the maximum concentrations observed outside the barrier (e.g., W2_6m). Benzene concentrations were the highest at W28_15m (0.126 mg/L), which coincided with low dissolved oxygen (DO) levels (1.86 mg/L) and elevated bicarbonate (HCO_3^-) concentrations, indicating that active degradation of the contaminant was occurring [25,26]. Similarly, studies have demonstrated that changes in groundwater chemistry significantly influence the mobility of pollutants and their degradation rates across different environments [27].

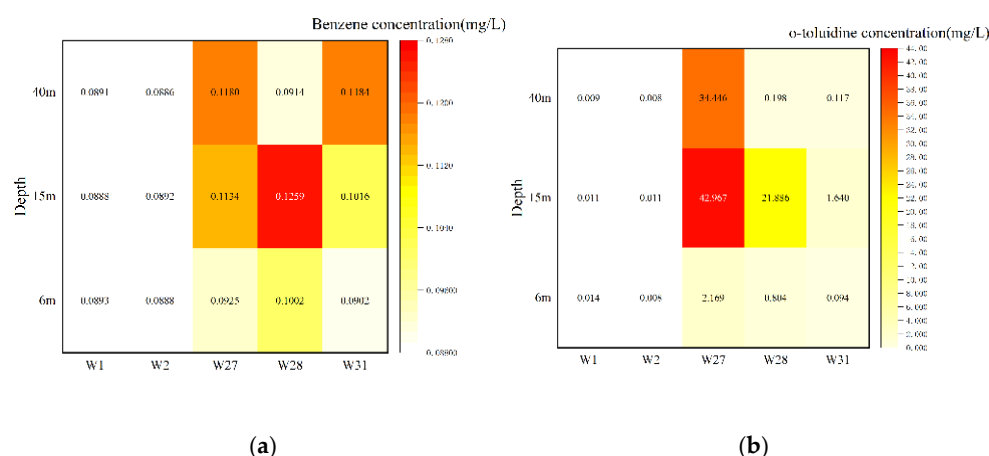


Figure 2. Characteristic contaminant concentration heat map: (a) benzene concentration; (b) o-toluidine concentration.

Aquifer heterogeneity also significantly affects the transport and distribution of pollutants. At our study site, the horizontal hydraulic conductivity at the 6 m and 15 m depths was relatively high (7.53×10^{-3} and 6.72×10^{-3} m/s), allowing contaminants to migrate more quickly in the horizontal direction. In contrast, at the 40 m depth, the horizontal conductivity dropped sharply to 7.53×10^{-6} m/s, indicating slower lateral pollutant movement. Similarly, vertical hydraulic conductivity was higher at 6 m (3.53×10^{-3} m/s) and 15 m (2.73×10^{-3} m/s), but significantly lower at 40 m (3.53×10^{-6} m/s). This difference in permeability suggests that contaminant transport is more restricted in the deeper layers. At the 6 m and 15 m depths, pollutants such as benzene and o-toluidine were detected at higher concentrations, reflecting more efficient horizontal and vertical transport. Conversely, at the 40 m depth, where permeability is lower, pollutant concentrations were comparatively lower, indicating reduced mobility in these layers.

3.2. Microbial Community Structure and Functional Diversity in Polluted and Unpolluted Zones

The abundance and diversity of microbial communities are important indicators for assessing the ecosystem health of water bodies [28]. The microbial diversity coverage of all monitoring wells in the study area was above 0.95, indicating that most of the microbial community diversity was captured. The composition of microbial communities at the phylum level and genus level at each sampling site is shown in Figures 3–6. As shown in Table 2, α diversity indices revealed significant differences in species richness and community diversity in different zones and depth samples. The shallow (6 m) layer of the non-polluted area (W1) exhibited the highest species richness (1015 OTUs), Shannon index (7.49), and Simpson index (0.98), which suggests that environments with lower pollution

pressures are favorable for the formation of resource-rich and stable ecosystems [29,30]. However, microbial diversity was significantly reduced in the polluted areas (W27, W28, W31). The Shannon index was only 2.37, the Simpson index was 0.58 in W27_15m, and the species richness was also reduced to 178 OTUs [31]. The higher pollutant concentrations (o-toluidine 42.97 mg/L, benzene 0.113 mg/L) significantly suppressed the community diversity of the polluted areas in W27_15m. The higher pollutant concentrations significantly suppressed community diversity while enriching specific degrading bacteria (*Acidovorax* and *Pseudomonas*), suggesting that pollution pressure is significantly selective for microbial communities [32,33]. Moreover, studies on bacterial consortia developed for BTEX degradation also show that the enrichment of specific microbial species like *Pseudomonas* was linked to the presence of toxic pollutants [34]. Specifically, *Acidovorax*, a genus often associated with the degradation of aromatic compounds, was enriched in the polluted zones [35]. Similarly, *Pseudomonas*, known for its ability to degrade a wide range of pollutants including hydrocarbons and aromatic compounds, was also dominant in the contaminated areas, reflecting the selective pressure of the pollutants [28,36].

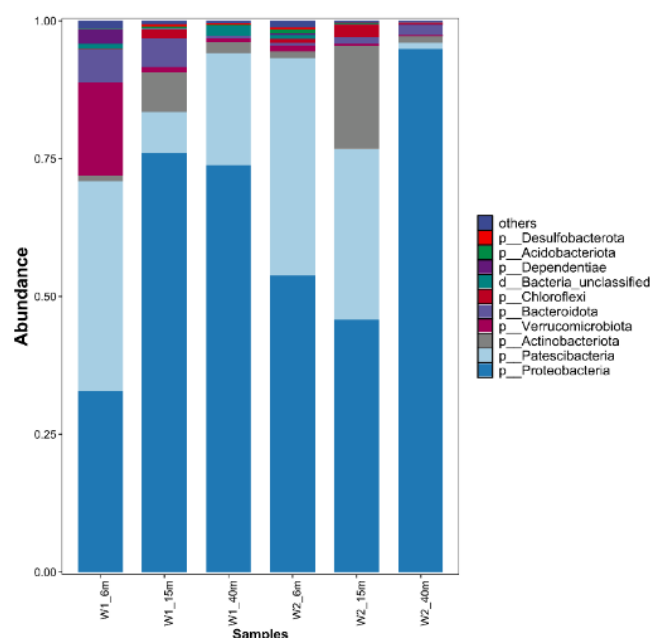


Figure 3. Composition of microbial communities at phylum level in non-contaminated area samples.

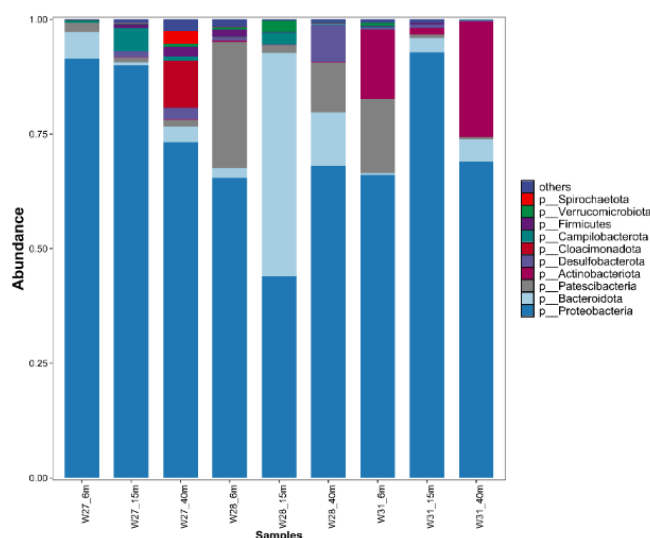


Figure 4. Composition of microbial communities at phylum level in contaminated area samples.

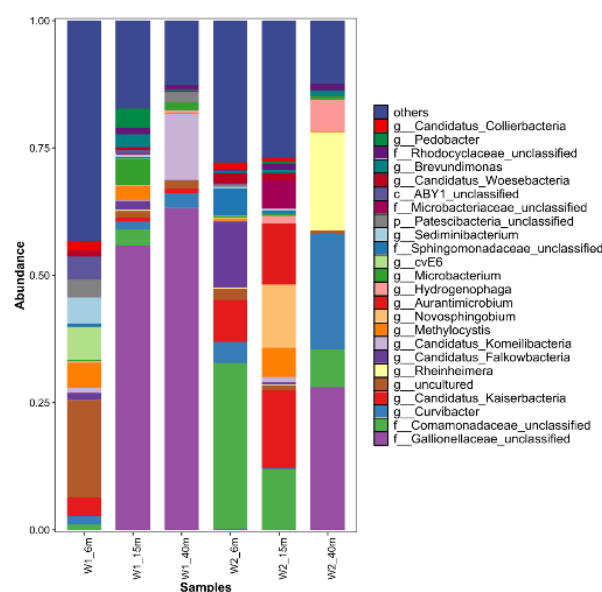


Figure 5. Composition of microbial communities at genus level in non-contaminated area samples.

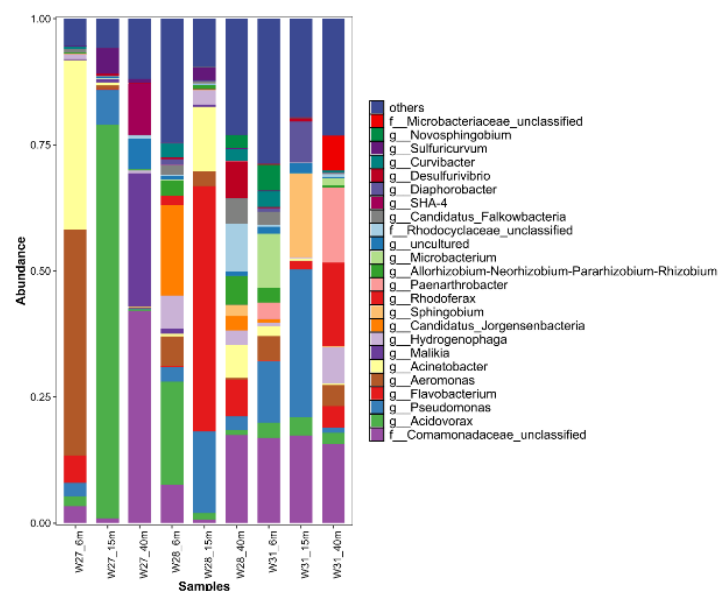


Figure 6. Composition of microbial communities at genus level in contaminated area samples.

Table 2. Index of alpha diversity of groundwater samples.

Sample	OTUs	Shannon	Chao	Simpson	ACE
W1_6m	1015	7.49	1030.9	0.98	1032.6
W1_15m	551	4.19	567.29	0.69	568.98
W1_40m	246	2.65	255.60	0.58	256.21
W2_6m	829	6.09	839.73	0.93	841.86
W2_15m	378	5.67	383.26	0.95	382.86
W2_40m	183	3.71	186.03	0.84	186.64
W27_6m	162	3.20	163.29	0.77	163.65
W27_15m	178	2.37	180.38	0.58	181.03
W27_40m	205	3.25	208.14	0.75	208.51
W28_6m	387	5.72	398.58	0.94	397.64
W28_15m	178	3.23	188.65	0.73	184.85
W28_40m	316	5.80	325.93	0.96	326.66
W31_6m	640	6.84	647.06	0.97	645.95
W31_15m	204	4.11	206.02	0.86	206.49
W31_40m	186	4.89	192.99	0.93	191.08

The depth variation of microbial communities also reflects the effect of environmental conditions on community distribution in different zones. In the non-polluted zone (W2), shallow samples (6 m) exhibited a high species richness (829 OTUs) and Shannon index (6.09); however, in the deeper layer (40 m), the OTUs were reduced to 183, and Shannon's index decreased to 3.71, suggesting that microbial diversity is limited by the nutrient-poor environment at the deeper level [37,38]. In contrast, W28 points within the contaminated area showed different depth distribution characteristics. The shallow (W28_6m) and middle (W28_15m) layers were enriched with aerobic degrading bacteria such as *Pseudomonas* and *Acinetobacter* [28,39]. In contrast, the microbial community in the deep layer (W28_40m) tended to be homogenized, suggesting that pollutant degradation in this region may be driven by a combination of metabolic pathways. In particular, the deeper community was enriched with flora capable of participating in anaerobic degradation (Gallionellaceae and *Candidatus Jorgensenbacteria*), reflecting the regional specificity of pollutant degradation [40,41].

As shown in Figure 7, the redundancy analysis (RDA) revealed significant relationships between environmental factors and microbial community composition across the groundwater samples from different zones (W1, W2, W27, W28, and W31). The first two axes (RDA1 and RDA2) explain 30.54% and 23.32% of the total variation, respectively, with a cumulative explanation of 53.86%, indicating that environmental factors significantly shape the microbial community structure in this study area [42].

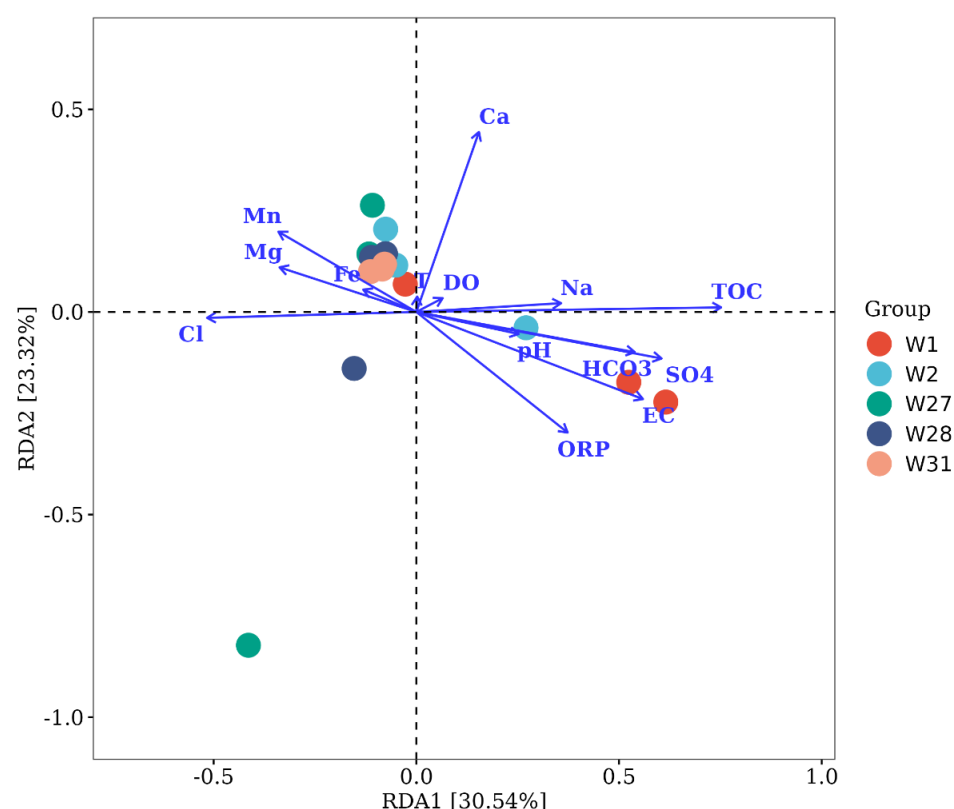


Figure 7. Redundancy analysis (RDA) of environmental factors influencing microbial community structure in groundwater samples.

The clustering patterns in the RDA plot reveal distinct environmental gradients associated with polluted (W27, W28, W31) and unpolluted zones (W1, W2). The polluted zones (W27, W28, and W31) are strongly associated with environmental variables such as HCO_3^- , SO_4^{2-} , EC, and pH, reflecting their critical roles in driving the microbial community structure under contamination stress [43]. Specifically, the high correlation of W27 with sulfate

indicates the presence of sulfate-reducing conditions, which are favorable for anaerobic metabolic processes and degradation pathways dominated by sulfate-reducing bacteria [44]. The close alignment of W28 with HCO_3^- and EC suggests that carbonate buffering and ionic strength are key drivers of microbial dynamics in this zone, particularly influencing aerobic and anaerobic degradation processes [45]. In contrast, unpolluted zones (W1 and W2) are closely associated with variables such as ORP, TOC, and DO. These associations highlight the dominance of aerobic microbial communities in these zones, supported by higher oxidizing conditions and nutrient availability [46]. The clustering of W1 around ORP and DO indicates that oxygen availability plays a significant role in maintaining microbial diversity and activity, promoting aerobic degradation mechanisms [47]. The deep layer of W31 is characterized by strong associations with Ca, Mg, and Mn, suggesting that these ions influence the microbial community structure in subsurface environments. The association of these variables with W31 highlights the potential role of metal-reducing bacteria and their contribution to pollutant degradation in anoxic conditions. Interestingly, Cl^- appears to be a distinguishing factor for W2, reflecting its role in shaping microbial communities in the unpolluted zones. The strong correlation with ORP in W2 further suggests that oxidative conditions in this zone facilitate the dominance of aerobic metabolic pathways [48].

The FAPROTAX analysis results further supported these findings, as shown in Figure 8. FAPROTAX is a tool suitable for predicting the functional annotations of biogeochemical cycling processes, particularly for elements such as carbon, nitrogen, phosphorus, sulfur, and hydrogen, in environmental samples like those from marine, freshwater, or terrestrial ecosystems. The analysis provides a robust prediction of microbial community functions, highlighting the importance of biogeochemical cycles in the degradation processes observed in this study [49,50]. The microbial communities in the non-polluted areas (W1, W2) mainly performed carbon and nitrogen cycle-related functions such as aerobic_chemoheterotrophy and nitrogen_fixation [47]. Especially at W1_6m, the high abundance of Methylocystis and Gallionellaceae reflected the diversity of ecological functions under aerobic and hypoxic conditions at this site [51,52]. In contrast, functions within the contaminated zone (W27, W28, W31) were significantly focused on pollutant degradation-related pathways. The shallow (W27_6m) community of W27 was enriched in aromatic compound degradation, driven by Acidovorax and Aeromonas, while the mesocosm (W27_15m) exhibited enhanced sulfate respiration, which corresponded to Desulfobacter enrichment [44]. In addition, the community in the deeper W31 layer (W31_40m) exhibited photoheterotrophy and sulfide metabolism, suggesting adaptive changes in the microbial community under pollution stress [39].

Overall, the microbial communities in the non-polluted and polluted areas showed significant differences in diversity, functional distribution, and environmental response. The non-polluted zones (W1, W2) exhibited high diversity and ecological health, whereas the polluted zones (W27, W28, W31) showed significant selective pressures, resulting in a decreased community diversity and functional concentration, on pollutant degradation. RDA analyses showed that HCO_3^- , SO_4^{2-} , and other environmental factors drove the distribution of degradation-associated flora in the polluted zone, while ORP and Cl^- dominated the distribution of aerobic functional flora in the non-polluted zone. Combined with the functional prediction, these results clearly reveal the complex regulatory mechanisms of environmental factors in pollutant degradation and microbial community function, which provide an important theoretical basis for the study of natural pollutant attenuation.

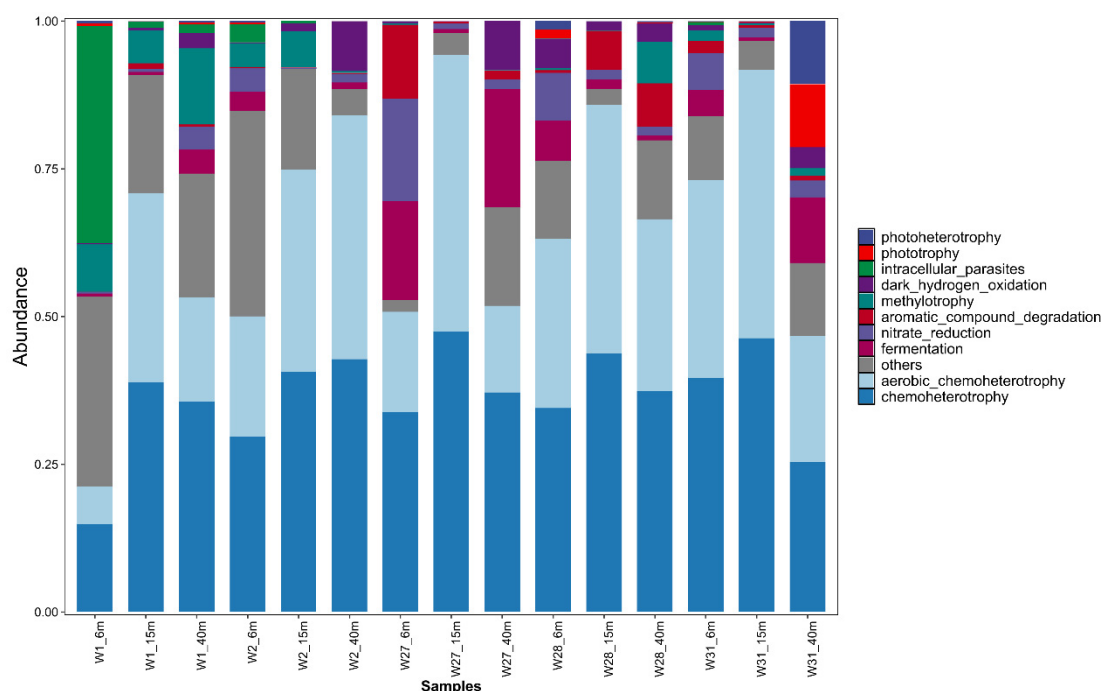


Figure 8. The community functions predicted by FAPROTAX for the groundwater samples.

3.3. Isotope Analysis Reveals Evidence of Natural Attenuation of Pollutants

Isotope analysis is an important tool for assessing the natural attenuation of pollutants, and the dynamic processes and mechanisms of pollutant degradation can be clarified by resolving hydrogen isotope (δD) and carbon isotope ($\delta^{13}C$) data (Table 3) at points in the contaminated area (W27, W28, W31) [53]. During microbial degradation, lighter isotopes are preferentially utilized, leading to the relative enrichment of heavier isotopes in residual contaminants [54]. Therefore, changes in isotope ratios serve as critical indicators of pollutant degradation. The fractionation effect of hydrogen isotopes reflects metabolic pathways such as water molecule cleavage, while carbon isotope variations are indicative of ring-opening and oxidative metabolic processes of aromatic compounds, and the combination of the two provides quantitative evidence of the degradation behaviors in the contaminated area [55]. De Vera et al. assessed the degradation of CBs and benzene in the groundwater of an industrial site located in the USA and found that a significant enrichment of ^{13}C occurred for CBs and benzene, with the greatest degree of enrichment in 1,2-DCB ($\sim 10\%$). This may be due to the relatively large differences in hydrogeochemical conditions and microorganisms between the two sites [56].

Table 3. Isotopic data of groundwater samples.

Sample	δD	$d(^{13}C_{\text{benzene}}/^{12}C_{\text{benzene}})$	$d(^{13}C_{\text{o-toluidine}}/^{12}C_{\text{o-toluidine}})$
W27_6m	−45.4	−31.76	−30.00
W27_15m	−44.6	−29.30	−28.39
W27_40m	−46.5	−35.04	−28.40
W28_6m	−46.1	−32.64	−30.27
W28_15m	−46.3	−34.86	−28.45
W28_40m	−41.6	−30.70	−31.99
W31_6m	−47.4	−34.23	−33.13
W31_15m	−44.5	−32.51	−29.50
W31_40m	−47.0	−33.60	−33.32

The study data showed that δD values varied from -47.4% to -41.6% within the polluted area, while carbon isotopes ($\delta^{13}C$) varied from -35.04% to -29.30% and from

−33.32‰ to −28.39‰ between benzene and o-toluidine, respectively. These variations showed significant spatial heterogeneity between different points and depths.

At point W27, the δD and $\delta^{13}C$ data reflect significant differences between shallow, intermediate, and deep layers. The $\delta^{13}C$ value for benzene in the shallow layer (W27_6m) was −31.76‰, while o-toluidine had a $\delta^{13}C$ value of −30.00‰, and the relatively low enrichment effect suggests that the degradation process is more active in the shallow layer [54]. However, in the middle layer (W27_15m), the enrichment effect of carbon isotopes was weakened (benzene: −29.30‰; o-toluidine: −28.39‰), while the deeper layer (W27_40m) showed the most significant carbon isotope fractionation (benzene: −35.04‰; o-toluidine: −28.40‰). Combined with the hydrogen isotope data (δD values varying from −45.4‰ to −46.5‰), it can be surmised that the degradation of pollutants in the deeper environment of W27 is somewhat limited, possibly due to the decrease in microbial activity under anaerobic conditions or the inhibitory effect of high pollution concentrations on the degrading bacteria [57]. The data from point W28, on the other hand, show different characteristics, especially the δD value of −41.6‰ in the deep layer (W28_40m), which was the most enriched point in the polluted area, and the carbon isotope variations (benzene: −30.70‰; o-toluidine: −31.99‰) further indicated that the pollutant degradation process was active at this point. This phenomenon may be related to the water chemistry conditions in the deeper layer of W28, e.g., the higher HCO_3^- concentration provided a rich carbon source for microorganisms, and the appropriate redox potential promoted the metabolism of aromatic compounds [58]. In contrast, although the shallow (W28_6m) and middle (W28_15m) layers showed some carbon isotope fractionation effects, the enrichment degree (shallow benzene: −32.64‰; middle benzene: −34.86‰) was slightly lower than that of the deeper layer, suggesting that pollutant degradation in the shallow and middle layers was somewhat limited. The isotope data at point W31 further reveal the complexity of pollutant degradation. The δD value of the shallow layer (W31_6m) was −47.4‰, while the $\delta^{13}C$ value of benzene was −34.23‰ and that of o-toluidine was −33.13‰, which showed a more significant fractionation effect, suggesting that the pollutants underwent an active degradation process in the shallow layer. However, in the deeper layer (W31_40m), although the δD values showed some enrichment (−47.0‰), the magnitude of carbon isotope variations was smaller (benzene: −33.60‰; o-toluidine: −33.32‰), suggesting that the deeper degradation activities were relatively weak. This may be related to the lower redox conditions in the deeper layers and the reduced diversity of microbial communities. Taken together, the δD and $\delta^{13}C$ data within the contaminated area show significant spatial and depth heterogeneity. Isotopic enrichment effects were most pronounced in the deeper layers of W28, suggesting that the contaminant degradation process was most active in this area, which may be dominated by highly efficient functional bacterial communities (*Acidovorax* and *Pseudomonas*). In contrast, the degradation activities in the deeper layers of W27 and W31 were limited, mainly by environmental conditions. The combined analysis of hydrogen and carbon isotopes reveals the degradation pathways and dynamic processes at different locations within the contaminated area, which provides a reliable basis for the quantitative assessment of the natural attenuation of pollutants and scientific support for the optimization of the remediation strategy of the contaminated site.

4. Conclusions

This study combined high-throughput sequencing, physicochemical analysis, and stable isotope technology to explore the natural attenuation process and driving mechanism of benzene and o-toluidine in groundwater in typical chemical-contaminated sites in southeastern China. The results showed that the degradation process of pollutants in the contaminated areas (W27, W28, and W31) was significantly affected by environmental

factors. HCO_3^- and SO_4^{2-} were the main driving factors affecting the distribution and degradation function of microbial communities, while ORP and Cl^- dominated the microbial community structure in the uncontaminated areas (W1 and W2). Stable isotope analysis further confirmed the heterogeneity of pollutant degradation. The deep layer (W28_40m) showed the strongest δD and $\delta^{13}\text{C}$ enrichment effects, indicating that there was the most active degradation process in this layer; meanwhile, the degradation process in the deep layers of W27 and W31 was limited, mainly due to anaerobic conditions and reduced microbial diversity. These findings clarify the degradation dynamics and driving mechanisms of different contaminated areas and provide a theoretical basis for the quantitative evaluation of natural attenuation, as well as practical guidance for the optimization of remediation strategies for contaminated sites.

In the process of contaminated site remediation, natural attenuation should be used as a low-intervention and cost-effective remediation technology, especially in sites with complex geological structures and slow pollutant decay rates. In particular, in sites with horizontal and vertical barriers, the attenuation efficiency should be regularly evaluated in combination with environmental factor monitoring and stable isotope analysis to ensure the scientificity and feasibility of the remediation strategy. In addition, this study emphasizes that in the remediation of contaminated sites, the distribution of microbial communities and environmental factors are closely related, and understanding the changing trends of these factors is crucial for predicting the pollutant decay process. Future research should continue to explore how to further improve the efficiency of natural attenuation through the adjustment of microbial community structure and the optimization of environmental factors, and provide empirical support for the management of similar contaminated sites.

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