

# Evaluation of the response of dynamic changes in the natural attenuation of chloride-polluted groundwater via high-throughput sequencing: degradation characteristics and environmental changes

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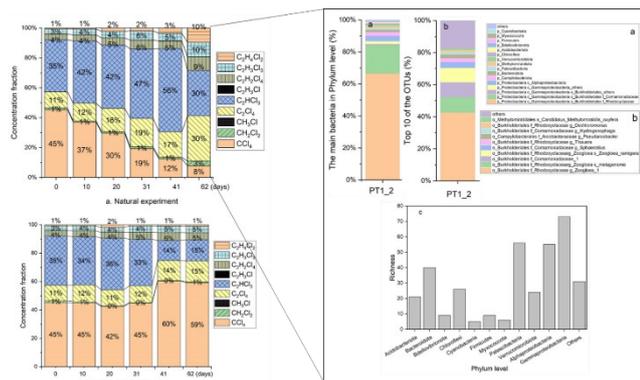
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## Graphical abstract



## Abstract

Due to its cost-effectiveness and absence of secondary pollution, the biological approach for eradicating chloride pollution has attracted widespread interest. However, numerous obstacles, including the limited presence of degrading microbes and a substantial impact from environmental variables arises during its practical application. The purpose of this study was to investigate the impact of ambient environmental factors during field surveys on the degradation of microorganism performance. The findings of this study demonstrate a negative correlation between the chlorinated hydrocarbons (CHCs) concentrations and pH, oxidation-reduction potential (ORP). These results suggest that redox conditions would improve the efficiency of chloride degradation. In addition, the degradation of CHCs was found to be primarily influenced by *Dechloromonas*, which accounted for > 1% of the total 16S rRNA sequences. In the absence of dechlorinating bacteria, the degradation efficiencies for CCl<sub>4</sub>, C<sub>2</sub>Cl<sub>4</sub>, and C<sub>2</sub>H<sub>2</sub>Cl<sub>4</sub> were 91.7%, 87.8%, and 86.0% lower than the efficiency in the presence of

these microorganism, respectively. Therefore, this study provides a comprehensive understanding of the role of biological degradation in controlling chloride pollution, along with substantial evidence supporting the extensive implementation of biological degradation technology.

**Keywords:** Natural attenuation, chloride pollution, environmental factor, dechlorinated bacteria, groundwater

## 1. Introduction

The rapid development of the urbanization and industrialization is always accompanied by organic matter pollution of water bodies, sediments, etc. The exploration of highly efficient methods is imperative in addressing the issue of pollution. To date, a range of biological treatments, including natural attenuation and membrane bioreactor, among others, have been implemented. These methods have demonstrated notable efficacy in removing pollutants and exhibit favorable environmental cost profiles. The utilization of membrane bioreactors for wastewater treatment has gained significant attraction due to its notable advantage of minimal sludge production (Menachem and Phillip 2011; Zhang *et al.* 2021). Whilst, severe membrane fouling poses a significant challenge to the filtration efficiency, leading to increased operational costs and hindering its potential for further application (Zhang and Jiang 2019). In contrast, natural attenuation can be used as a low-cost remediation technique for addressing water pollution, contaminated sediments, and contaminated soils that have restricted migration capabilities (Nalok *et al.* 2022; Xue *et al.* 2022). Natural attenuation processes may enhance the stability of contaminants or decrease their mass, volume, mobility, and toxicity (Chapelle and Bradley 1998; Damikouka and Katsiri 2020; Fu *et al.* 2022). Natural attenuation is a sluggish process comprised of physical, chemical, and

biological processes. As a result, it is susceptible to various factors, and can persist for an extended period of time and undergo hydrological variations (Mulligan and Yong 2004; Zhang *et al.* 2019). The efficacy of natural attenuation is also contingent upon the physicochemical characteristics of sediments and the water column. Consequently, it is essential to estimate the potential mobility and transportation of pollutants, which are influenced by environmental conditions and factors, such as pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), and microbes, etc., in the surrounding environment (Damikouka and Katsiri 2020; Cappuyens and Swennen 2008; Kelderman and Osman 2007). This understanding is necessary to comprehend the degradation mechanism of pollutants and develop a cost-effective strategy to enhance their removal efficiency.

Chlorinated hydrocarbons (CHCs) compounds are widely used in chemical applications as cleaning solvents, lubricants, heat transfer fluids, spot removers, plasticizers, degreasing agents and cleaning fluids.  $\text{CCl}_4$  is one of the most commonly used among the chlorinated organic compounds. Furthermore, it is an important contaminant because of its environmental persistence and carcinogenicity (Hamonts *et al.* 2009; Puigserver *et al.* 2020; Xu *et al.* 2015). In addition,  $\text{CCl}_4$  is difficult to be reduced in groundwater through self-purification, which is of great concern as a significant environmental contaminant for drinking water quality (Altalyan *et al.* 2016). It is commonly found in water environments, including groundwater and surface water, at concentrations that exceed the Environmental Protection Agency's (EPA) drinking water standard for  $\text{CCl}_4$  of 5  $\mu\text{g/L}$ . In aerobic environments, reductive dechlorination of  $\text{CCl}_4$  sequentially generates  $\text{CH}_2\text{Cl}_2$ ,  $\text{CH}_3\text{Cl}$ , etc., and  $\text{CO}_2$ , eventually. Microbes can utilize  $\text{C}_2\text{H}_2\text{Cl}_2$  or  $\text{C}_2\text{H}_3\text{Cl}$  as a sole carbon source (Schiefler *et al.* 2018; Weatherill *et al.* 2018). However, it is difficult to demonstrate that contaminants are biodegrading in situ as opposed to being reduced by physical processes such as volatilization, dilution or sorption to sediment. Due to the diffusion of contaminants, the measurement of the decrease in CHC concentrations over time as a result of biodegradation was impeded (Eggleton and Thomas 2004; Gaza *et al.* 2019; Longe and Enekwechi 2007). Recently, the isotope analysis was used to estimate the degradation caused by non-degradative dissipative processes (Hermon *et al.* 2018; Liu *et al.* 2018; Nijenhuis *et al.* 2007; Pooley *et al.* 2009).

At a number of industrial sites, improper use and disposal practices have resulted in widespread contamination of water masses with CHCs, which has a negative impact on the daily lives and productivity of local residents. Current researches are centered on determining how to restore a polluted environment and select cost-effective and efficient governance measures. Several scholars have studied in the laboratory the mechanism of microbial CHCs degradation (Dutta *et al.* 2022; Liu *et al.* 2020; Shi *et al.* 2020; Wang *et al.* 2021; Xu *et al.* 2015). However, little is understood about the biodegradation of CHCs in contaminated groundwater in situ (Witt *et al.* 2002). In this

study, we selected a chloride-polluted site to measure the degradation of chloride with and without the addition of  $\text{HgCl}_2$  over a two-month period, analyzed the diversity and distribution of bacteria associated with CHCs biodegradation, and investigated the potential degradation mechanism. According, this study is likely to contribute to the environmental remediation of groundwater chloride contamination.

## 2. Methods and materials

### 2.1. Study area

The sampling site was situated in close proximity to a small-scale chemical plant in Linyi, which was located in the southernmost region of Shandong Province. The pollution of surrounding groundwater caused by seepage pit discharge is directly responsible for the presence of dead fish in the fishing grounds. The horizontal migration of pollutants diffuses gradually from north to south gradually along with the flow direction of groundwater. Based on the previous investigations into groundwater pollution, it is imperative to repair and control groundwater pollution immediately.

### 2.2. Experimental design

The polluted water samples were pumped from an aquifer at a depth of  $\sim 20$  m through the well at the PT1-2 station, which was located downstream of the discharge point. Before laboratory analyses, the samples were stored in brown glass bottles sealed with a grey butyl septum and an aluminum crimp seal, then placed in a dry, cool environment. In this study, two groups were established: natural and  $\text{HgCl}_2$  addition. The addition of  $\text{HgCl}_2$  aims to eliminate microbial activity and investigate the degradation of CHCs pollution in the absence of microorganism. Every  $\sim 10$  days, water samples were taken from the bottles to determine the changes in CHCs and the related environmental factors with and without the microbial activity. Every group was measured in triplicates.

### 2.3. Chloride detection

In this study, the CHCs, including  $\text{CCl}_4$ ,  $\text{CH}_2\text{Cl}_2$ ,  $\text{CH}_3\text{Cl}$ ,  $\text{C}_2\text{Cl}_4$ ,  $\text{C}_2\text{HCl}_3$ ,  $\text{C}_2\text{H}_3\text{Cl}$ ,  $\text{C}_2\text{H}_2\text{Cl}_4$ ,  $\text{C}_2\text{H}_3\text{Cl}_3$  and  $\text{C}_2\text{H}_4\text{Cl}_2$ , were detected using gas chromatography-mass spectrometry (GC-MS) as the procedure described by Wang *et al.* (2021). The chromatographic column in GC-MS comprised a DB-5MS capillary column. He was used as the carrier gas, and the constant flow mode was implemented. Without shunt injection, the column flow rate was 1.5 mL/min, and the automatic injection volume was 1  $\mu\text{L}$ . The temperature of the injector was 280  $^\circ\text{C}$ . The heating procedure of gas chromatography was as follows: initially, the column temperature was set at 50  $^\circ\text{C}$  for a duration of 4 min. Subsequently, the temperature was raised to 210  $^\circ\text{C}$  for 1 min at a rate of 12  $^\circ\text{C}$  per min. This was followed by a further increase to 250  $^\circ\text{C}$  for a period of 3 min with a heating rate of 5  $^\circ\text{C}$  per min. Finally, the temperature was elevated to 275  $^\circ\text{C}$  for 5 min at a rate of 0.5  $^\circ\text{C}$  per min, and then further increased to 300  $^\circ\text{C}$  for 5 min at a rate of 15  $^\circ\text{C}/\text{min}$ . Regarding the Time-of-flight mass spectrometry parameters, the ion source temperature was set at 250  $^\circ\text{C}$ , the ionization mode was electron bombardment ionization

(70 eV), and the scanning ion range was 50 to 550. The frequency of data acquisition was 20 Hz.

#### 2.4. Environmental factor

As for the determination of environmental factors, we selected dissolved Fe (DFe), dissolved Mn (DMn), pH, DO, ORP, alkalinity and nutrients (nitrate and sulphate) of the subterranean water to investigate their effect on the natural attenuation of CHCs.

The DFe and DMn were determined by flame atomic absorption spectrometry (iCE 3300 AAS, Thermo Scientific™). The pH, DO and ORP value was determined using an S975-uMix multiparameter tester (METTLER TOLEDO). DO: 0–99 mg/L, 0.001 mg/L,  $\pm$  10%. ORP: -2000 mv–2000 mv, 1 mv,  $\pm$  0.1 mv).

The alkalinity (ALK) of water was determined using acid-base indicator titration in accordance with the water and wastewater monitoring and analysis method. Firstly, phenolphthalein was used as an indicator and titrated with HNO<sub>3</sub> standard solution until the solution changed from red to colorless. Next, methyl orange was used as the indicator and continued to titrate with the same concentration of HNO<sub>3</sub> solution until the solution changed from orange yellow to orange red. The ALK was calculated based on the amount of HNO<sub>3</sub> used.

The water samples for nutrient measurement were stored in pre-cleaned Nalgene bottles (Wang *et al.* 2022). Duplicate samples of 1 L were filtered through a 0.22  $\mu$ m polycarbonate membrane (PC, Millipore). The filtrate was collected in a polypropylene bottle, sealed with Parafilm®, and frozen (-20 °C) prior to analysis. Nitrate (NO<sub>3</sub><sup>-</sup>) was analyzed utilizing colorimetric techniques and a Lachat QuickChem 8500 autoanalyzer (Lachat Instruments, USA). Additionally, the sulphate (SO<sub>4</sub><sup>2-</sup>) in water was analyzed by ion chromatography using the method of precipitation with BaCl<sub>2</sub>. The detection limits were 0.002 mg/L for NO<sub>3</sub><sup>-</sup>, 0.003mg/L for SO<sub>4</sub><sup>2-</sup>, respectively.

#### 2.5. DNA extraction and high-throughput sequencing

The extraction of environmental DNA followed the protocol outlined by Wang *et al.* (2020) and Xue *et al.* (2021). Before the high-throughput sequencing, environmental DNA was lyophilized and stored in -80 °C for long-term preservation following extraction. The DNA quantity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

For each DNA sample, two rounds of PCR amplification were performed (primers: 341F and 805R) with V3-V4 as the hypervariable regions. High-throughput sequencing of 16S rRNA genes was performed at Magigene (Magigene Biotechnology, Guangzhou, China) on an Illumina Nova6000 platform (paired-end 250-bp mode), according to the manufacturer's guidelines. The raw sequencing data obtained in this study is accessible through the GenBank database under the accession number PRJNA576689. The procedures were based on the works of Shi *et al.* (2020) and Xue *et al.* (2021).

#### 2.6. Sequence analyses

The raw sequence reads were processed with the QIIME 2 (version 2019.1) program (Bolyen *et al.* 2019). Generally, the total of reads was retrieved after demultiplexing. Then, q2-DADA2 was applied for quality control, filtering, denoising, merging, and chimera removal from these sequences. A Naïve Bayes classifier artifact was applied to assign the ASVs to taxa at 99% using the Silva classifier data set. In order to minimize the sampling effects, the depth of the original OTU table was reduced to 60,000 sequences per sample. QIIME 2 was also used to generate alpha-diversity metrics including observed OTUs.

#### 2.7. Statistical analysis

Two degradation experiments were conducted in parallel. Statistical tests were performed by the package 'pwi' in R software. Differences were considered significant at the 0.05 level. Pearson's correlation analysis was conducted using the Statistical Package for Social Sciences program (version 19.0).

The relationships between environmental factors and chloride concentration were analyzed using redundancy analysis (RDA) with the vegan R package (version 3.6.0). The analysis was calculated using the original data on environmental variables and chloride concentrations. The statistical significance of the RDA model was evaluated using ANOVA. This analysis could aid in determining the most influential factors and the extent to which chloride pollution degradation has been impacted by bacterioplankton composition.

### 3. Results

#### 3.1. Degradation of chloride contamination with and without HgCl<sub>2</sub> addition

Natural attenuation is an essential method for minimizing chloride contamination. Microorganisms play a critical role in the progress of natural attenuation (Damikouka and Katsiri 2020; Zhang *et al.* 2019). To figure out the microorganism activity in the natural attenuation of chloride, we inhibited the microorganism with HgCl<sub>2</sub> in our study.

According to the data presented in Figure 1a and Table 1, the degradation of CCl<sub>4</sub> was found to be more prominent in the natural group than that in the HgCl<sub>2</sub> addition group. Over a period of two months, there was a notable reduction in the concentration of CCl<sub>4</sub> from 920  $\mu$ g/L to 34.3  $\mu$ g/L. The addition of HgCl<sub>2</sub> had no discernible impact on the levels of CCl<sub>4</sub> concentrations, as evidenced by the negligible change in concentration from 920  $\mu$ g/L to 847  $\mu$ g/L. This suggests that microorganisms play a substantial role in the natural attenuation of CCl<sub>4</sub>. Akin to CCl<sub>4</sub>, C<sub>2</sub>H<sub>2</sub>Cl<sub>4</sub> and C<sub>2</sub>H<sub>3</sub>Cl<sub>3</sub> showed a similar downward trend in natural groups (Figures 1e and 1f). Over a period of two months, the concentration of C<sub>2</sub>H<sub>2</sub>Cl<sub>4</sub> decreased from 77  $\mu$ g/L to 39.2  $\mu$ g/L, and the concentration of C<sub>2</sub>H<sub>3</sub>Cl<sub>3</sub> decreased from 68.8  $\mu$ g/L to 42.5  $\mu$ g/L. While the concentrations of C<sub>2</sub>H<sub>2</sub>Cl<sub>4</sub> and C<sub>2</sub>H<sub>3</sub>Cl<sub>3</sub> decreased by less than 10% in the HgCl<sub>2</sub> addition groups.

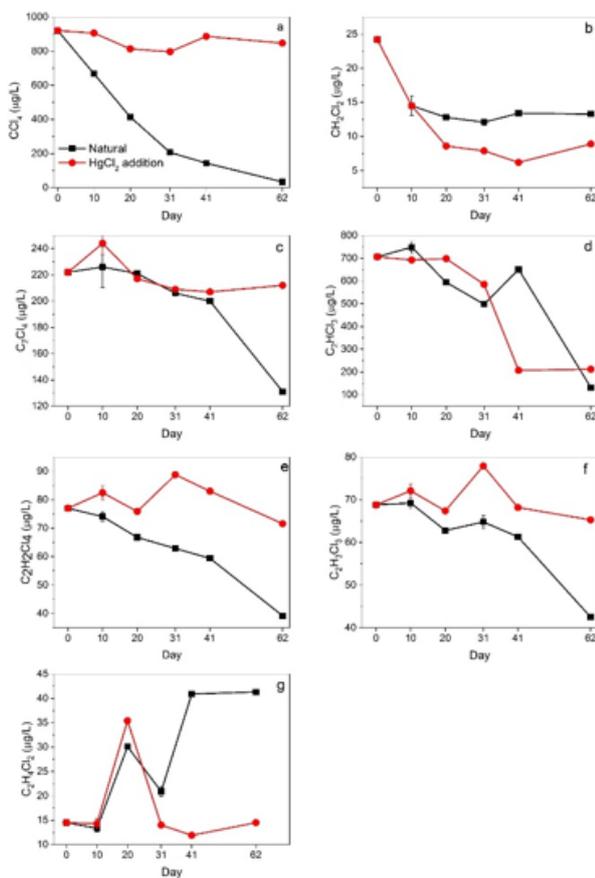
In contrast, the CH<sub>2</sub>Cl<sub>2</sub> exhibited a more pronounced decrease in the HgCl<sub>2</sub> addition groups (Figure 1b and Table

1). The  $\text{CH}_2\text{Cl}_2$  concentrations decreased from 24.2  $\mu\text{g/L}$  to 6.2  $\mu\text{g/L}$  during the first 41 days and then increased to 8.9  $\mu\text{g/L}$  by the end. In the natural group, the concentrations of  $\text{CH}_2\text{Cl}_2$  decreased sharply during the first ten days and

then remained relatively high, with a slight upward trend. So, the initial decline in  $\text{CH}_2\text{Cl}_2$  was not entirely caused by microbial activity.

**Table 1.** The changes of CHCs contents (unit:  $\mu\text{g/L}$ ) in the natural and  $\text{HgCl}_2$  addition experiments

CHCs ( $\mu\text{g/L}$ )	natural group (days)						$\text{HgCl}_2$ addition group (days)					
	0	10	20	31	41	62	0	10	20	31	41	62
$\text{CCl}_4$	920	667.5	414	206.5	144	34.3	920	906	813	797	887	847
$\text{CH}_2\text{Cl}_2$	24.2	14.5	12.8	12.1	13.4	13.3	24.2	14.5	8.6	7.9	6.2	8.9
$\text{CH}_3\text{Cl}$	0	0	0	0	0	0	0	0	0	0	0	0
$\text{C}_2\text{Cl}_4$	222	226	221	206	200	131	222	244	217	209	207	212
$\text{C}_2\text{HCl}_3$	706	748.5	594	498	651	131	706	692.5	698	585	207	212
$\text{C}_2\text{H}_3\text{Cl}$	0	0	0	0	0	0	0	0	0	0	0	0
$\text{C}_2\text{H}_2\text{Cl}_4$	77.0	74.1	66.8	62.9	59.4	39.2	77.0	82.5	75.9	88.8	83.0	71.6
$\text{C}_2\text{H}_3\text{Cl}_3$	68.8	69.25	62.8	64.8	61.3	42.5	68.8	72.1	67.4	77.9	68.2	65.3
$\text{C}_2\text{H}_4\text{Cl}_2$	14.5	13.35	30.1	20.9	40.9	41.3	14.5	14.3	35.4	14	11.9	14.5



**Figure 1.** Degradation of chlorinated hydrocarbons in natural and  $\text{HgCl}_2$  addition experiments (a:  $\text{CCl}_4$ , b:  $\text{CH}_2\text{Cl}_2$ , c:  $\text{C}_2\text{Cl}_4$ , d:  $\text{C}_2\text{HCl}_3$ , e:  $\text{C}_2\text{H}_2\text{Cl}_4$ , f:  $\text{C}_2\text{H}_3\text{Cl}_3$ , g:  $\text{C}_2\text{H}_4\text{Cl}_2$ . Unit:  $\mu\text{g/L}$ )

In contrast to  $\text{CCl}_4$  and  $\text{C}_2\text{H}_2\text{Cl}_4$ , the concentration of  $\text{C}_2\text{Cl}_4$  (Figure 1c and Table 1) did not vary significantly with or without the addition of  $\text{HgCl}_2$  during the first 41 days. However, in the natural group, the concentration of  $\text{C}_2\text{Cl}_4$  decreased sharply by the end of the experiment (~41.0%). In addition, the concentrations of  $\text{C}_2\text{H}_4\text{Cl}_2$  in these two groups did not differ significantly during the first 20 days (Figure 1g). Then, the concentrations of  $\text{C}_2\text{H}_4\text{Cl}_2$  in the experimental groups supplemented with  $\text{HgCl}_2$  exhibited a significant increase compared to the natural group. From Figure 1d, the  $\text{C}_2\text{HCl}_3$  contents showed a downward trend

over the course of the experiment. Furthermore, no discernible difference was observed between the two groups at the end of experiment.

### 3.2. The environmental changes

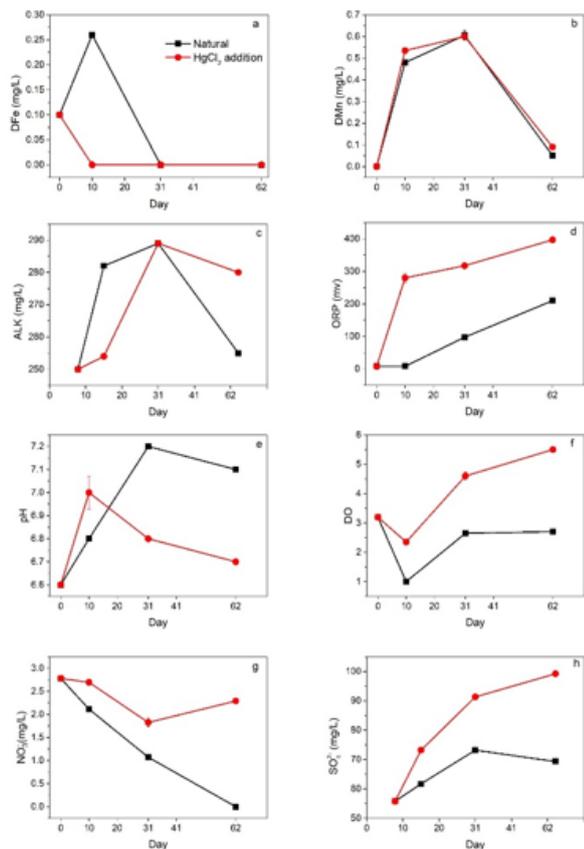
The microbial activity, which is regulated by environmental factors, will invariably have an effect on the natural attenuation of chloride contamination in the groundwater. According to Figure 2a, it can be observed that the concentrations of dissolved iron (DFe) exhibited an initial increase from 0.10 mg/L to 0.26 mg/L over a period of 10 days, then decreased to zero in the subsequent natural experiment, whereas the DFe concentrations in the  $\text{HgCl}_2$  addition group fluctuated rapidly to zero. Unlike DFe, the levels of DMn were found to be similar between the natural group and the  $\text{HgCl}_2$  addition group, indicating that the presence of DMn does not have a significant impact on microbial activity (Figure 2b).

In both the natural group and the  $\text{HgCl}_2$  addition groups, the alkalinity (Figure 2c) increased by 1.2-fold over the course of one month. At the end of the experiment, the alkalinity in the natural group decreased to 255 mg/L, which is close to the initial value (250 mg/L). Conversely, the alkalinity level in the  $\text{HgCl}_2$  addition group (280 mg/L) did not display any significant alteration.

All types of biochemical reactions normally occur and complete within a specific redox range in biological cells. The environments with a low redox potential are unquestionably crucial for anaerobic microorganisms (Wang *et al.* 2021). In both the natural and  $\text{HgCl}_2$  addition experiments, the ORP values exhibited an increase over a period of two months (Figure 2d). In the natural groups, the ORP increased (~26 folds) considerably less than in the  $\text{HgCl}_2$  addition groups, which the ORP rose from 8 mv to 397 mv (~50 folds).

The pH and DO are also essential variables for microbial activity (Figure 2e and 2f). In the natural group, the pH increased from 6.6 to 7.1, whereas in the  $\text{HgCl}_2$  addition group, the pH increased to 7 and then decreased to 6.7, approaching the initial pH (6.6). The DO values between the two groups differ significantly. The  $\text{HgCl}_2$  addition group consistently has a higher DO than the natural group. The

DO values dropped from 3.2 mg/L to 2.4 mg/L before rising to 5.5 mg/L at the end of the HgCl<sub>2</sub> addition group. The DO value dropped precipitously from 3.2 mg/L to 1.0 mg/L before recovering to 2.7 mg/L, which is also lower than the initial DO concentration of 3.2 mg/L.



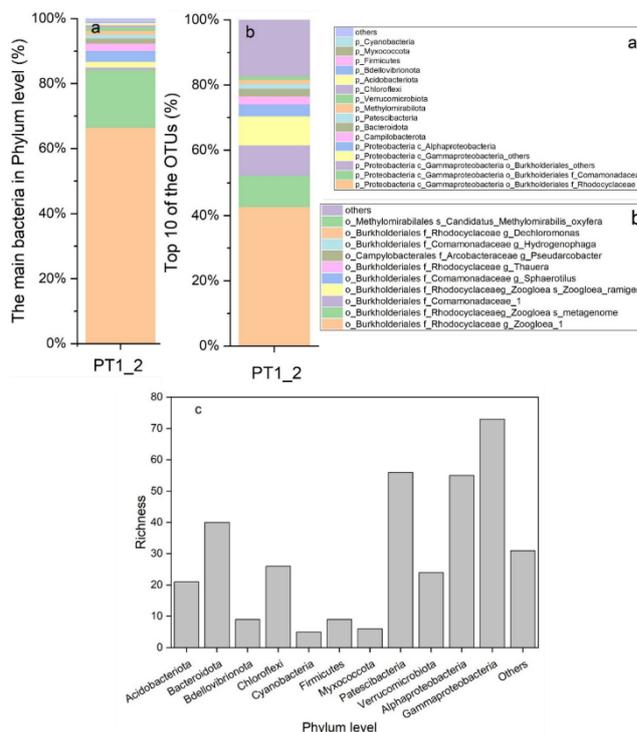
**Figure 2.** The changes of DFe (a, mg/L), DMn (b, mg/L), alkalinity (ALK, c, mg/L), ORP (d, mv), pH (e), DO (f, mg/L), NO<sub>3</sub><sup>-</sup> contents (g, mg/L) and SO<sub>4</sub><sup>2-</sup> contents (h, mg/L) in natural and HgCl<sub>2</sub> addition experiments.

The nutrients may also be the most influential factors in microbial activity (Figures 2g and 2h). Over a period of two months, the concentration of NO<sub>3</sub><sup>-</sup> in the natural groups exhibited a decline from 2.78 mg/L to complete absence. The presence of NO<sub>3</sub><sup>-</sup> did not exhibit a discernible decline in response to the addition of HgCl<sub>2</sub>, indicating that the microbial activity was effectively suppressed. The levels of SO<sub>4</sub><sup>2-</sup> exhibited divergent trends. In these two groups, SO<sub>4</sub><sup>2-</sup> concentrations increased over the course of two months. In HgCl<sub>2</sub> addition groups, the concentration of SO<sub>4</sub><sup>2-</sup> exhibited a notable increase from 55.8 mg/L to 99.2 mg/L, surpassing the magnitude of increase observed in the natural group.

### 3.3. The microbial structure

The groundwater microbial communities were dominated by Gammaproteobacteria, which accounted for over 86% of the total 16S rRNA sequences in our sample. The most abundant family of Gammaproteobacteria is Rhodocyclaceae. *Dechloromonas*, a chloride degradation genus, was accounted for 1.22% of the total sequences. The relative abundance of Alphaproteobacteria, Campilobacterota, Bacteroidota, Patescibacteria,

Methylomirabilota and Verrucomicrobiota varied from 1.0% to 3.3%. *Proteobacteria* and *Bacteroides* are typical microorganisms for organic pollutants degradation. *Proteobacteria* are capable of degrading a wide range of organic pollutants and reducing the biological toxicity of pollutants (Lee *et al.* 2010). *Bacteroidetes* are involved in the degradation of polymers and complex organic substances and are capable of decomposing dead cells containing polysaccharides and proteins into simple organic molecules (such as ethanol and lactic acid) that can be metabolized by other species (Gomez-Pereira *et al.* 2012). Typically, Patescibacteria participate in anaerobic fermentation and are associated with the decomposition of organic matter (Wei *et al.* 2022). The relative abundance of the remaining microbial groups in our sample is less than 1%. Specifically, the proportion of cyanobacteria in our sample was found to be less than 0.6% (Figure 3a). These top 10 abundant OTUs were mainly affiliated to Burkholderiales, which accounts for nearly 80% of the total microbial relative abundances in our sample, including the *Zoogloea*, *Comamonadaceae*, *Thauera*, and *Dechloromonas* subgroups (Figure 3b). *Comamonadaceae* is a family that degrades denitrifiers and transforms sulfur (Khan *et al.* 2002). *Thauera* is the potential organic pollutant degrading bacteria from phenol degrading bacteria. Additionally, Gammaproteobacteria was not only the most abundant group in our samples but also the most diverse among the sample analyzed (Figure 3c).



**Figure 3.** The structure of bacteria in the underground water (a: Abundance of the top 10 OTUs. b: Abundance of the main bacteria in Phylum level. c: The richness of the main bacteria in Phylum level).

## 4. Discussion

### 4.1. Chlorinated hydrocarbons and their metabolites

The extents of reductive dechlorination of CCl<sub>4</sub>, C<sub>2</sub>Cl<sub>4</sub> and C<sub>2</sub>H<sub>2</sub>Cl<sub>4</sub> were evaluated by comparing the ratio of each

contaminant to the total concentrations of CHCs within their respective biodegradation pathways.

The sharp decrease in  $\text{CCl}_4$  fractions, declining from 45% to 8%, indicates the indigenous microbial community is capable of anaerobically metabolizing  $\text{CCl}_4$  via reductive dechlorination. Low concentrations of  $\text{CH}_2\text{Cl}_2$  and  $\text{CH}_3\text{Cl}$  were detected, with the fractions increasing from 1% to 3% for  $\text{CH}_2\text{Cl}_2$  and <1% for  $\text{CH}_3\text{Cl}$ , respectively, by the end of the experiment. In comparison to  $\text{CH}_3\text{Cl}$ , which accounted for <1% of the total  $\text{CCl}_4$ ,  $\text{CH}_2\text{Cl}_2$  was the principal metabolite of  $\text{CCl}_4$  reductive dechlorination and accounted for 2% to 39% of the total  $\text{CCl}_4$ , indicating the degradation of  $\text{CCl}_4$ . This demonstrated natural attenuation, which is regulated by microorganism activities, is an appropriate remediation strategy for chloride-contaminated groundwater (Munro *et al.* 2017; Wright *et al.* 2017).

However,  $\text{C}_2\text{Cl}_4$  increased from 11% to 30% in the course of the experiment. The metabolite,  $\text{C}_2\text{HCl}_3$ , increased from 35% to 56% within the first 41 days, surpassing the concentration of the matrix. Subsequently, it decreased to 30%, which coincided with the percentage observed for  $\text{C}_2\text{Cl}_4$ .

Similar to  $\text{C}_2\text{Cl}_4$ ,  $\text{C}_2\text{H}_2\text{Cl}_4$  fractions increased from 4% to 9%, and  $\text{C}_2\text{H}_2\text{Cl}_4$  metabolites ( $\text{C}_2\text{H}_3\text{Cl}_3$  and  $\text{C}_2\text{H}_4\text{Cl}_2$ ) were detected in low concentration and accounted for <10%. The fractions of  $\text{C}_2\text{H}_3\text{Cl}_3$  and  $\text{C}_2\text{H}_4\text{Cl}_2$  increased from 3% and 1% to 10%, respectively. The observation indicates that the natural attenuation of  $\text{C}_2\text{H}_2\text{Cl}_4$  was not evident and that a portion of the  $\text{C}_2\text{H}_3\text{Cl}_3$  and  $\text{C}_2\text{H}_4\text{Cl}_2$  originated from other chlorides.

In the course of  $\text{HgCl}_2$  addition experiments, it was observed that the fractions of  $\text{CCl}_4$  increased from 45% to 59%, the fractions of  $\text{C}_2\text{Cl}_4$  increased from 11% to 15%, and the fractions of  $\text{C}_2\text{H}_2\text{Cl}_4$  increased from 4% to 5%. This demonstrated that the  $\text{CCl}_4$ ,  $\text{C}_2\text{Cl}_4$  and  $\text{C}_2\text{H}_2\text{Cl}_4$  did not degrade upon the addition of  $\text{HgCl}_2$ .

#### 4.2. Analysis of environmental changes during the degradation of chloride

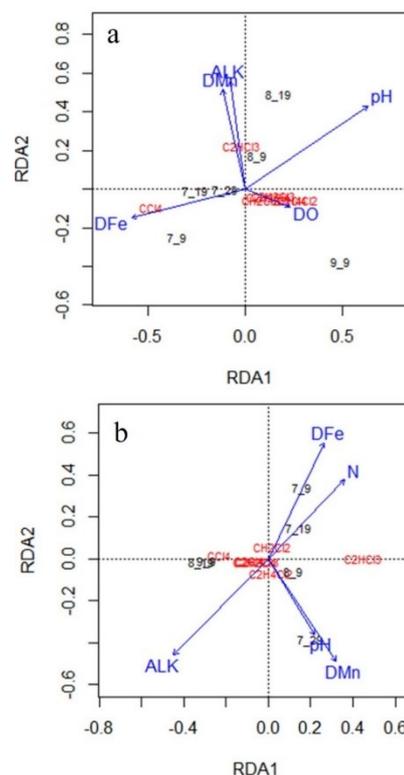
To better analyze the environmental factors which would affect the degradation of chloride, we conducted spearman correlation analysis and redundancy analysis (RDA plot, Figure 4) using chloride data ( $\text{CCl}_4$ ,  $\text{CH}_2\text{Cl}_2$ ,  $\text{CH}_3\text{Cl}$ ,  $\text{C}_2\text{Cl}_4$ ,  $\text{C}_2\text{HCl}_3$ ,  $\text{C}_2\text{H}_3\text{Cl}_3$ ,  $\text{C}_2\text{H}_2\text{Cl}_4$ ,  $\text{C}_2\text{H}_3\text{Cl}_3$ ,  $\text{C}_2\text{H}_4\text{Cl}_2$ ), as well as environmental factors (DFe, DMn, ALK, pH, DO,  $\text{NO}_3^-$  (N), ORP,  $\text{SO}_4^{2-}$  (S)).

In a natural experiment, the degradation of these chlorinated hydrocarbon organics exhibited significant negative correlations with ORP, particularly for  $\text{CCl}_4$ ,  $\text{C}_2\text{Cl}_4$  and  $\text{C}_2\text{H}_2\text{Cl}_4$  ( $r = -0.99$ ,  $p < 0.001$ ). Additionally,  $\text{C}_2\text{HCl}_3$ ,  $\text{C}_2\text{H}_3\text{Cl}_3$  and  $\text{C}_2\text{H}_4\text{Cl}_2$  decreased significantly as ORP increased ( $p < 0.05$ ). The ORP in the groundwater or sediment would influence the microbial activity that controls the natural attenuation of chloride contamination (Chandrasekhar *et al.* 2021; Fu *et al.* 2021).

$\text{CCl}_4$ ,  $\text{C}_2\text{Cl}_4$  and  $\text{C}_2\text{H}_2\text{Cl}_4$  showed negative relationships with pH ( $r = -0.81$ ,  $p < 0.05$ ,  $r = -0.75$ ,  $p = 0.08$ , and  $r = -0.81$ ,  $p < 0.05$ , respectively). The decrease in pH would inhibit the

activity of bacteria that promote the degradation of chlorinated hydrocarbon organics. In contrast to pH,  $\text{CCl}_4$  and  $\text{C}_2\text{H}_2\text{Cl}_4$  showed a weakly positive correlation with DFe concentrations ( $r = 0.76$ ,  $p = 0.08$ ), while  $\text{C}_2\text{Cl}_4$  demonstrated a significantly positive correlation with DFe ( $r = 0.88$ ,  $p < 0.05$ ). This is further supported by the higher DFe concentrations, which, as a result of the lower pH, aided in the natural attenuation of chloride contamination (Czinnerová *et al.* 2020).

Both ALK and DMn exhibited similar effects on the degradation of  $\text{CH}_2\text{Cl}_2$ . However, there is no relationship between these two environmental factors and other chlorinated hydrocarbon organics. The  $\text{CH}_2\text{Cl}_2$  concentration decreased as ALK and DMn concentration increased slightly ( $p = 0.07$ ).

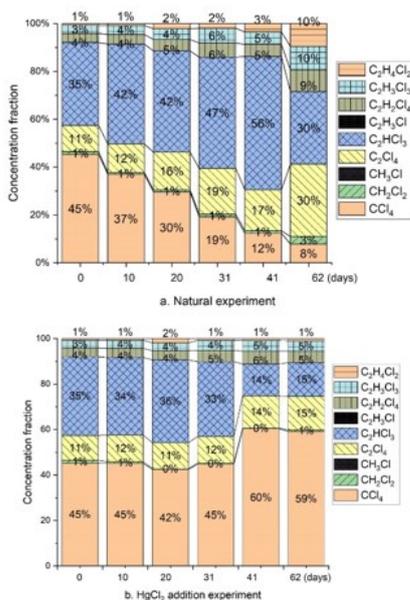


**Figure 4.** The RDA plots in the natural (a) and  $\text{HgCl}_2$  addition (b) experiments.

The RDA ordination explained 98% of the variation in environmental factors and established a clear trend in the natural attenuation of chlorinated hydrocarbon organics (Figure 4). In the RDA plot, the horizontal axis (RDA 1) explained 84.0 % of the variance, and the most influential environmental variables were pH, DFe and DO, which may be associated with the environmental redox potential. The vertical axis (RDA 2) explained 14.8% of the variance, and the environmental variables that contributed the most were ALK, DMn and pH. Samples from different months (July, August and September) were clearly separated in different quadrants. Variation partitioning analysis revealed that  $\text{CCl}_4$  degraded over time and that its natural attenuation was affected by the DFe and pH in a manner consistent with the results of spearman's correlation analysis. The mechanistic study also implied that the degradation of CHCs was mainly induced the  $e_{\text{aq}}^-$  which is associated with the redox environment (Wang *et al.* 2021).

In the experiment involving the addition of  $\text{HgCl}_2$ ,  $\text{CCl}_4$  demonstrated a slight negative correlation with DMn and ALK, and a positive correlation with N content ( $p = 0.07$ ). Conversely, the metabolic  $\text{CH}_2\text{Cl}_2$  demonstrated a negative correlation with ALK and a positive correlation with N content ( $p = 0.02$ ).  $\text{C}_2\text{Cl}_4$  concentrations also demonstrated a negative correlation with ALK, DO ( $p < 0.05$ ), and ORP, S ( $p = 0.07$ ), and a positive correlation with N content ( $p < 0.05$ ). Its metabolic  $\text{C}_2\text{HCl}_3$  was negatively correlated with ORP, S ( $p < 0.05$ ) and ALK, DO ( $p = 0.07$ ), and positively correlated with N content ( $p = 0.07$ ). These results indicated that these environmental factors have the potential to impact the natural attenuation of  $\text{CCl}_4$  and  $\text{C}_2\text{Cl}_4$ , even in the absence of biological degradation. Redox process could potentially contribute to the natural attenuation. Compared to the natural experiment, the addition of  $\text{HgCl}_2$  effectively impeded the natural attenuation of  $\text{C}_2\text{H}_2\text{Cl}_4$ , thereby indicating that biological degradation may be the predominant mechanism responsible for the natural attenuation of  $\text{C}_2\text{H}_2\text{Cl}_4$ .

The RDA ordination accounted for 97.8% of the variance in environmental factors. In the RDA plot (Figure 5), the horizontal axis (RDA 1) accounted for 94.7% of the variance, while the vertical axis (RDA 2) accounted for 3.1% of the variance. Although ALK, DFe, N and DMn contributed significantly to the two axes, chloride did not achieve high RDA 1 or RDA 2 scores. Because the biological degradation of chloride would be inhibited by the addition of  $\text{HgCl}_2$ , the natural attenuation of chloride was greatly reduced. Some studies have also shown that biotic processes play a critical role in the degradation of chlorinated benzene, as evidenced by the works of Han *et al.* (2021) and Puigserver *et al.* (2020).



**Figure 5** Concentration fractions of chlorinated hydrocarbons and metabolites in 2 months (a. natural experiment, b.  $\text{HgCl}_2$  addition experiment).

## 5. Conclusion

Due to its proven efficacy and cost-effectiveness, the implementation of in situ bioremediation has emerged as

a promising strategy for the detoxification of contaminated sites. The distribution of ambient redox processes has a direct bearing on the efficiency and capacity of bioremediation in groundwater systems. In this study, the degradation of chlorides from contaminated groundwater was primarily attributable to the redox environment. Additionally, the pH value and DFe content were found to have a significant impact on the dechlorination of CHCs. By controlling groundwater conditions such as pH, DFe, ORP, this treatment proved to be highly effective in fostering a biodegradable environment. This can be used to gain a comprehensive understanding of the efficacy of natural attenuation, as well as the relative significance of multiple simultaneously occurring field-scale attenuation processes.

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## Competing interests

The authors declare no competing of interest.

## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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