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Thermally Enhanced Biodegradation Mechanisms of Trichloroethene and Benzene Co-contaminants in Groundwater: Insights into Microbial Functional Gene Enrichment and Biogeochemistry

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Abstract

A series of thermally enhanced biodegradation (TEB) experiments at varying temperatures (15, 30 and 45 °C) were conducted to evaluate their effects on the biodegradation kinetics of trichloroethene (TCE) and benzene co-mingled contaminants using indigenous microbial consortiums. Results demonstrated that the highest TCE biodegradation rate, 0.35 μmol L^{-1} day^{-1}, with a half-life of 49 days, was observed at 30 °C, whereas the biodegradation of benzene was enhanced to a less extent. The functional gene analysis results revealed that increased *tceA* and *tmoA* genes contributed to enhanced TCE and benzene biodegradation at 30 °C, respectively. Additionally, the reduced *vcrA* and *bvcA* abundance and lack of effective electrons may attribute to the *cis*-1,2-dichloroethylene stalling and low concentration of ethene. Moreover, the biogeochemical analyses confirmed that elevated temperature promoted limited denitrification and sulfate reduction processes, which expedited methanogenesis, leading to the enhanced dechlorination of TCE. Additionally, the abiotic degradation of TCE facilitated by FeS, evidenced by acetylene and ethene detections, also contributed to the observed enhanced TCE degradation. The study shed lights on the role of temperature in enriching microbial functional gene and influencing biogeochemical conditions for the simultaneous anaerobic biodegradation of TCE and benzene. The findings demonstrated that optimized thermal treatments can effectively bioremediate chlorinated and aromatic hydrocarbons in groundwater.

Keywords: Thermally enhanced biodegradation; trichloroethene; benzene; degradation rate; functional genes

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1. Introduction

Monoaromatics, e.g., benzene, toluene, ethylbenzene, and xylene (BTEX), and chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are widely used as industrial solvents and resulted in mixed contamination in soils and groundwater due to their improper disposal or leakage, posing serious threat to ecosystems and public health [1, 2]. Biodegradation was believed as an effective and eco-friendly strategy for removing CAHs and BETX. Previous studies were conducted to evaluate the biodegradation of mixed CAHs and BTEX under aerobic conditions, in which BTEX are oxidized and TCE can be degraded via co-metabolism [3]. However, the co-contamination of BTEX and CAHs was commonly observed with 142 and 405 mg/L, respectively, under anoxic or anaerobic conditions in subsurface environments [1]. While the individual biodegradation of BTEX or CAHs has been studied extensively [4, 5] under anaerobic condition, the simultaneous biodegradation of these mixed contaminants, particularly under thermally enhanced conditions, need further investigation.

Yet the bioremediation of CAHs in soil and groundwater are commonly carried out under anaerobic conditions, in particular with the addition of biostimulants (e.g., ethanol and molasses), to achieve the methanogenesis conditions [6, 7]. Previous studies have confirmed the reductive dechlorination of parent chloroethenes, e.g., PCE and TCE, to non-toxic ethene, driven by microbial dechlorinators such as *Dehalococcoides mccartyi* (*Dhc*) and *Dehalogenimonas* (*Dhg*) via organohalide respiration [8-13]. In addition, there were also relevant studies reported the degradation of benzene in anaerobic condition. As reported previously by Liang et al. [14], benzene can be anaerobically transformed in a methanogenic enrichment culture and produce hydrogen and acetate, which can facilitate the reductive dechlorination as electron donors, indicating the potential possibility for co-biodegradation of both TCE and benzene. Moreover, the anaerobic biodegradation pathways for benzene have also been elucidated under conditions of denitrification, sulfate reduction, and iron reduction, with intermediates formed such as benzoate and phenol and further metabolized to CO_2 and CH_4 [15, 16]. During these processes, benzene was degraded as electron donor and it has been reported that functional genes of *tmoA* and catechol 2,3-dioxygenase gene (*cat2,3*), responsible for initial attack and meta-cleavage of benzene-ring via oxidation pathway [17]. However, the biodegradation behaviours and mechanisms of BTEX and chloroethenes in co-mingled plume under anaerobic condition have not been fully investigated.

Temperature plays a crucial role in modulating microbial activities and contaminant degradation rates [18]. For examples, it was reported that when the temperature was increased to ~30 ℃, the activity and growth of various *Dehalococcoides* populations were promoted, and thus the dechlorination of cDCE and vinyl chloride (VC) was enhanced [19]. It was also reported that cell growth increased 4-fold by increasing the temperature from 20 to 35 ℃, and then decreased rapidly at 36-40 ℃ [18]. Although some studies have shown that microbial dechlorination rates increase significantly with temperature [20-22], the specific impact of elevated temperatures on indigenous microbial communities collected from the co-contaminated sites and their capacity to degrade co-contaminants have not been thoroughly explored. Previous reports indicated that the biodegradation of BTEX and chloroethenes were more active between 10 °C and 30 °C, with diminished efficacy at higher temperatures [23, 24]. Temperature affects the acclimation period of microorganisms, leading to the selection of distinct populations, influencing the physiology and diversity of the microbial consortium, thereby affecting the bioremediation of contaminants [25]. Laboratory studies were conducted by Zeman et al. [23] to evaluate the temperature impacts on the anaerobic biotransformation of BTEX and it was found that the composition and activity of microbial communities shifted in a temperature-dependent manner. A field study revealed that the total number of reductive dehalogenase genes (*vcrA* and *bvcA*) and diversity of dechlorinating bacteria (e.g., *Dehalococcoides mccartyi* and *Dehalobacter* sp.) increased after heating the treated zone from 17 to 30 ℃ [26]. When the temperature increased from 15 to 30 ℃, the abundance of *vcrA* remains static or declined, and the abundance of *bvcA* gene continuously increased, whereas the opposite relationship was observed when the temperature was over 35 ℃ [27]. Thermally enhanced biodegradation (TEB), referring to thermal treatment with elevated temperatures and biodegradation, has been shown to be promising in promoting microbial growth and metabolic activities with isolated strains or enriched cultures [28], yet its effects on indigenous microbial communities remain largely unexplored.

To address the above knowledge gap, this study aims to evaluate the effectiveness of TEB for the remediation of TCE and benzene co-contaminants using indigenous microbial communities from co-contaminated soil without enrichment procedure. The key objectives are to (i) determine the biodegradation pathways and kinetics of mixed TCE and benzene at elevated temperature conditions, (ii) assess how temperature influences the microbial ecology and functional bacteria

and genes, and (iii) investigate the underlying mechanisms of enhanced biodegradation for co-contaminants under elevated temperatures. This research seeks to provide new insights into the potential of TEB as a viable approach for treating complex subsurface contaminations.

2. Materials and methods

2.1 Microcosm experiment set up

Microcosm experiments were conducted to assess TCE and benzene degradation at different temperatures using soils collected from a pesticide manufacturing site in Suzhou, Jiangsu Province, China. Soils, mainly consisting of silty clay, were sourced from 10 m below ground surface (bgs). The soil samples were air-dried and sieved, and no volatile organic compounds, including TCE, *cis*-1,2-dichloroethylene (cDCE), vinyl chloride (VC), and benzene, were detected in soil after pre-treatment. In addition, pesticides, including atrazine and dichlorvos, were under their respective detection limits in soil samples. Following the established methods for reductive dechlorination [9], approximately 15 g of soil, 72 mL of medium, and 0.1 mL of mixed TCE-benzene stock solution, using acetone as the solvent were added to 120 serum bottles, maintaining specific solid-liquid (5-50%) and headspace ratios (1-1.6:1) [29]. In addition, the target concentrations of TCE and benzene used in microcosm experiments were 6.55 mg/L for TCE and 60 mg/L for benzene, taking the following factors into consideration: (1) the initial contamination levels of TCE and benzene in groundwater were 4 mg/L and 14 mg/L, respectively, and (2) concentrations should be sufficiently high to account for the potential losses due to volatilization, especially under elevated temperature conditions. Bottles were sealed with butyl rubber septa and aluminium caps. The detailed method for establishing anaerobic conditions was provided in **Text S1**.

Previous studies have reported that the optimal temperature for reductive dechlorination of chlorinated ethenes such as TCE occurs at 25-30 °C [24, 30], and biodegradation can cease at ~45 °C [22], but the dechlorination can resume in the cooling period after thermal treatment of~40 °C [24]. In addition, it has been reported that the optimal temperature for microbial activities of benzene biodegradation ranges from 20-35 °C [31] and benzene biodegradation can still occur at 45 °C [32]. Therefore, for the thermal treatment in the current study, 15 °C was chosen to represent

the ambient groundwater temperature, which ranged from 16 °C to 18 °C in groundwater at the time of soils sampling, and 30 and 45 °C were selected to evaluate the impacts of thermal enhancement for the concurrent biodegradation of both TCE and benzene. Biotic treatments were incubated at 15 °C, and 30 °C and 45 °C, labelled as T-15, T-30, and T-45, while the abiotic sterilized treatments were also set up at each temperature (S-15, S-30, and S-45) to assess the loss of TCE and benzene due to volatilization. Moreover, the blank sample before thermal treatment without TCE and benzene co-contaminants dosage was labelled as CK. The microcosm experiments were carried out in two phases. In phase I, triplicates samples in different treatment groups were placed in water baths and incubated in the dark without shaking for 150 days, and then adjusted to room temperature (25 ± 3 °C) and lasted for 340 days in Phase II, totaling 490 days. Samples were collected periodically to measure TCE and its daughter products (cDCE, VC, ethene, acetylene), benzene and CH_4, CO_2, and for microbial and sediment analysis.

2.2 Determination of degradation of TCE, benzene and their intermediate products

TCE and its intermediate metabolites (cDCE and VC), along with benzene in liquid samples, were analysed every two months using a Shimadzu GC-2010 Plus equipped with an FID detector and a VF-624 column (60 m × 0.25 mm × 1.4 μm). Helium served as the carrier gas at 1 mL/min. The detector temperature was 250 °C, and the column temperature was held at 40 °C for 2 minutes, ramped to 90 °C at 6 °C/min (held for 2 minutes), then increased to 230 °C at 15 °C/min. Ethene and acetylene in headspace, indicators of complete biotic reductive dechlorination and abiotic degradation of TCE, respectively, were measured using the same GC-2010 Plus with an FID detector and a PLOTQ column (30 m × 0.32 mm × 20 μm) following methods previously described in our previous study [33]. Intermediate by-products of benzene biodegradation including toluene, phenol, and benzoate, were quantified with liquid samples by the purge-trap GC-MS with a DB-624 column (60 m × 0.25 mm × 1.4 μm) using helium (1.0 mL/min) as the carrier gas, at the end of TEB. The oven was programmed from 35 °C (held for 2 minutes) to 120 °C at 5 °C/min, then to 220 °C at 10 °C/min. The injection temperature was set at 220 °C, and the ion source was maintained at 280 °C.

CO_2 and CH_4 in headspace were measured with an Agilent GC-7890 equipped with an FID detector and a methanizer using a Porapak Q column (1 m × 0.125 in × 2 mm) [34, 35]. The detector

temperature was 250 °C, with hydrogen and air flow rates of 50 mL/min and 450 mL/min, respectively, using nitrogen as the carrier gas at 22.5 mL/min.

The total organic carbon (TOC) and the concentrations of sulfate, sulfite, nitrate, nitrite, and Fe species (total Fe, Fe(II), Fe(III)) in liquid phase in biotic treated samples (T-15, T-30, and T-45), and CK samples were measured at the end of incubation to assess biological processes during TCE and benzene degradation. Further details on the analytical methods are provided in Supporting Information **Text S2**.

2.3 DNA extraction, Illumina sequencing and quantitative real-time PCR (qPCR)

DNA from treated samples (T-15, T-30, and T-45) incubated at different temperatures for 490 days, along with the microbial community control in CK at the beginning of incubation, was extracted from 5 mL of cultures using a commercial DNA extraction kit, following the manufacturer's instructions. Triplicate samples were used for each treatment. Universal 16S rRNA primers (338F and 806R) were employed for PCR amplification, following protocols from a previous study [33]. The amplified DNA was purified using an AxyPrep DNA Gel Recovery Kit and sequenced on an Illumina MiSeq platform at Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Sequencing reads were deposited in the NCBI Sequence Read Archive with the project number PRJNA1173567. Image analysis, base calling, and error estimation were conducted using the Illumina Analysis Pipeline Version 2.6.

The qPCR was applied for quantifying the abundance of total bacterial 16S rRNA gene, *Dhc* 16S rRNA gene, *Dhg* 16S rRNA gene, RDase genes (*tceA*, *vcrA*, *bvcA*) and benzene biodegradation functional genes (*tmoA*, *cat2,3*) and the primers were shown in **Table S1 [33]**. The detailed description of 16S sequencing and protocols for qPCR were shown in **Text S3**.

2.4 Data analysis

The zero-order kinetic model was applied to explore the biodegradation of TCE (Eq. 1) and generation of its byproduct cDCE (Eq. 2) and first-order kinetic model for benzene (Eq. 3) as shown below:

$$\frac{dC_t}{dt} = -k_0 \leftrightarrow C_t = C_0 - k_0 \cdot t \quad (1)$$

$$\frac{dC_t}{dt} = k_0 \leftrightarrow C_t = k_0 \cdot t \quad (2)$$

$$\frac{dC_t}{dt} = -k_1 \cdot C_t \leftrightarrow In\frac{C_0}{C_t} = k_1 \cdot t \quad (3)$$

where C_0 is the initial concentration for biodegradation of TCE and benzene, C_t is the concentration of TCE, cDCE and benzene at time t. It is worth noting that C_0 and C_t concentrations were those of measured adjusted with consideration of the volatilization compared with abiotic treatments (S-15, S-30 and S-45). Moreover, k_0 is the zero-order kinetic rate constant for degradation of TCE and formation of cDCE, and k_1 is the first-order kinetic rate constant for degradation of benzene.

All samples were analysed in triplicate. Alpha diversity indices were calculated from amplicon sequence variants (ASVs) using QIIME2. Beta diversity was evaluated via Principal Coordinates Analysis (PCoA), based on Bray-Curtis distance matrices at the ASV level. Both alpha and beta diversity analyses were performed on the Majorbio Cloud Platform (www.majorbio.com). Correlation analyses between genes, degradative bacteria, and biodegradation kinetics were conducted using the Vegan package in R, and the results were presented as a correlation heatmap. One-way analysis of variance (ANOVA) was applied to test for significant differences in microbial alpha diversity and gene abundance, with a significance threshold of p=0.05.

3. Results and discussion

3.1 Thermally enhanced biodegradation of TCE and benzene co-contaminants

3.1.1 Degradation kinetics of TCE and benzene under TEB

The degradation of TCE and benzene under TEB followed zero-order and first-order kinetics, respectively (**Figure 1**). The rate constants (k_0 and k_1) and half-life ($t_{1/2}$) were presented in **Table 1**. The highest TCE biodegradation rate, 0.35 μmol L^{-1} day^{-1}, was observed at 30 °C, which was 2.5 and 5.6 times greater than those at 15 °C and 45 °C, respectively. This finding agrees with

previous study that identified the optimal dechlorination temperature for TCE as between 25 °C and 35 °C [36]. In T-45, the inhibited dechlorination in Phase I during incubation at 45 °C suggested that higher elevated temperature (45 °C) could cause damage to the microbial cell [24], leading to a lag of biodegradation. Yet later their activities were resumed in Phase II, as evidenced by the production of cDCE. However, after cooling to room temperature during Phase II, degradation of TCE resumed, albeit at a slower rate (0.062 μmol L^{-1} day^{-1}), which was still lower than those observed at 15 °C and 30 °C. These results suggested that mesophilic and thermophilic microorganisms in the native microbial community contribute to TCE degradation across a wide temperature range, as reported in mixed cultures [30, 37].

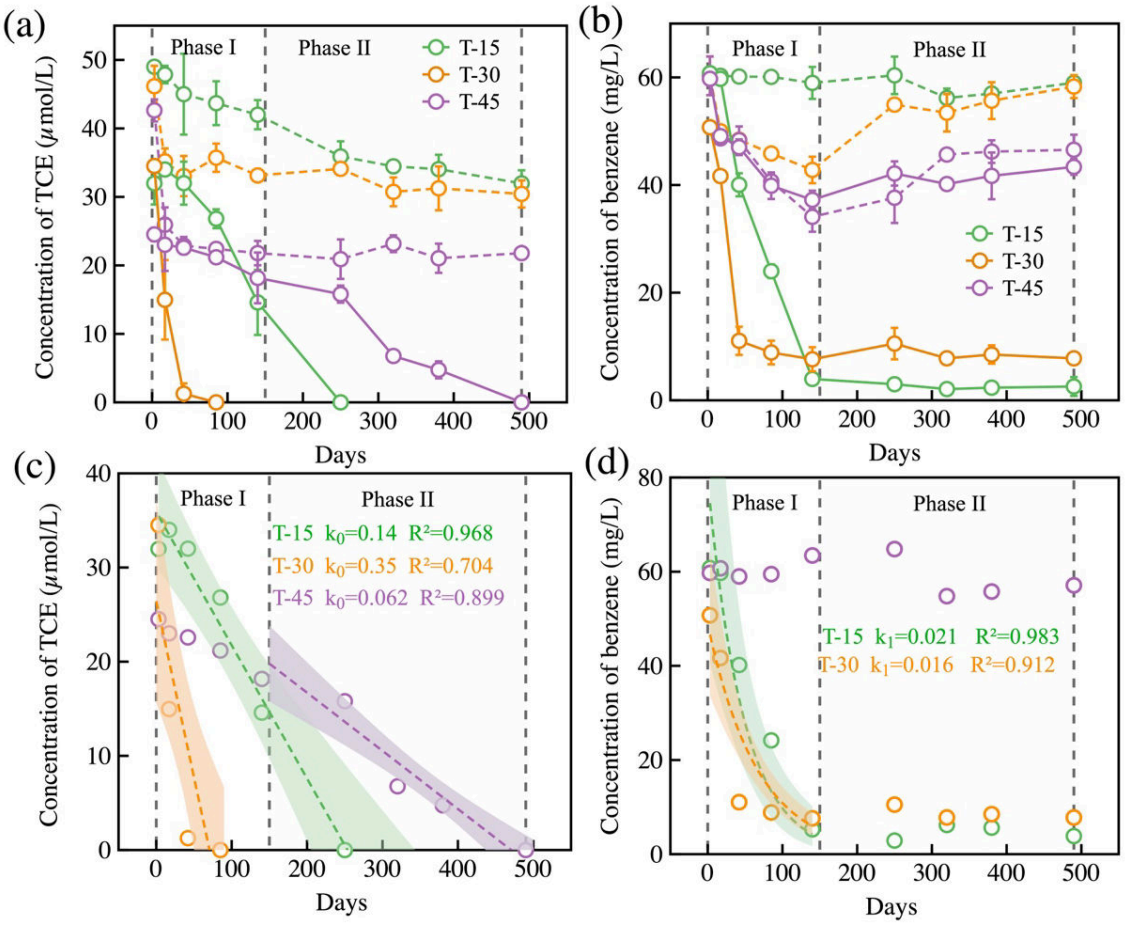

Figure 1. Degradation of TCE and benzene co-contaminants under TEB (Phase I: incubation at temperatures of 15 °C, 30 °C and 45 °C; Phase II: incubation at room temperature; Solid line in a, b: thermal treatment; Dash line in a, b: sterilized treatment) and their degradation kinetics (c, d,

dash line: Kinetic fitting curve, confidence = 95%). It is worth noting that the concentration losses due to volatilization (as illustrated in the control groups) were deducted from those in biotreatment groups at different temperature for kinetic fitting (c and d). Therefore, the kinetic parameters estimated were for biodegradation only.

Table 1. TCE and benzene biodegradation kinetics under different temperature

Contaminant	Treatment	Zero-order kinetics: $C_t = C_0 - k_0 \cdot t$			
		C_0 (μmol/L)	k_0 (μmol L^{-1} day^{-1})	$t_{1/2}$ (day)	R^2
TCE	T-15	31.97	0.14	114	0.968
	T-30	34.51	0.35	49	0.704
	T-45	24.52	0.062	240	0.899
Contaminant	Treatment	First-order kinetic: $In\frac{C_0}{C_t} = k_1 \cdot t$			
		C_0 (mg/L)	k_1 (day^{-1})	$t_{1/2}$ (day)	R^2
Benzene	T-15	59.73	0.021	33	0.983
	T-30	60.29	0.016	43	0.912
	T-45	59.25	/	/	/

$t_{1/2}$: The half-life ($t_{1/2}$) was calculated when C_t is $C_0/2$.

/: indicated that there was no biodegradation of benzene observed.

Benzene degradation occurred at both 15 °C and 30 °C, following first-order kinetics with rate constants (k_1) of 0.021 day^{-1} at in T-15 °C and 0.016 day^{-1} in T-30 °C (**Table 1**). Although a higher benzene biodegradation kinetics was observed in in T-30 °C, the overall benzene removal in T-15 °C eventually reached a lower concentration by Day 140, which persisted throughout Phase II, suggesting that a quick start in the biodegradation rate does not guarantee a higher overall bioremediation efficiency. Even though the benzene removal efficiency reached to 85.0% to 95.2% in T-15 and T-30 (Table S2), respectively, the residual concentrations of benzene remained at a few mg/L levels, suggesting that addition measures, such as the addition of electron acceptors (e.g., sulfate as reported by Cunningham et al. [38], is warranted in order to meet the cleanup goals. Moreover, no biodegradation of benzene was observed at 45 °C, and the similar magnitudes of benzene removal efficiency in T-45 and S-45 suggested benzene removal at this temperature was due to volatilization contribution rather than microbial activity (**Table S2**). The inhibited benzene biodegradation at 45 °C in this study indicated that 45 °C exceeded the thermal tolerance of the native benzene-degrading bacteria, causing irreversible cellular damage [39]. This finding was different from that observed by Zehnle et al. [40], who reported that thermophilic microorganisms could degrade benzene at 50 °C and 70 °C. In addition, it was found that benzene degradation was

highly enhanced at 30 °C and 45 °C compared to 15 °C in our previous study, with the co-contamination of benzo[a]pyrene [32]. The inconsistency might be attributed to the presence of TCE and its daughter products, which could pose toxicity to the microorganisms in the study community, influencing the activity of benzene degrading bacteria [41]. However, due to the lack of TCE or benzene standalone treatment groups in the current study, future studies are warranted to explicitly clarify the influences of co-contaminants on their respective biodegradation cannot be explicitly determined. In addition, the distinct indigenous environmental conditions where the microbial communities originated, suggested the complex interactions and responses to thermal treatment.

Furthermore, the volatilization under evaluated temperature contributed to the removal of both TCE and benzene (**Table S2**), and the volatilization contributions of TCE and benzene in S-45 were the highest, up to 48.8% and 41.6%, respectively. These results provided further insights into the application of TEB for TCE and benzene co-contaminants, which can be coupled with soil vapor extraction as a strategy to enhance the overall remediation efficiency. A detailed discussion on the degradation pathways will follow, further exploring the mechanisms behind thermal treatment and enhancing our understanding of TEB.

3.1.2 Daughter products and indicators microbial activities under TEB

During the TEB of TCE and benzene, cDCE, the intermediate product of TCE reductive dechlorination, was quantified and its production profiles were presented in **Figure 2a**. The formation of cDCE followed the zero-order kinetics (**Figure 2b**), and the consistent patterns for degradation of TCE and formation of cDCE, implied that reductive dechlorination to cDCE was the primary pathway for TCE degradation during the TEB process in this study. However, the formation rate of cDCE was lower than the TCE biodegradation kinetics, which was primarily due to the uncounted volatilization of cDCE to the headspace while the evaporation of TCE was accounted by the loss to the headspace in the control group, as noted in **Figure 1**. In addition, ethene was detected at low concentrations, albeit no VC was detected, indicating that cDCE may be further biodegraded, contributing slightly to the lower formation rate of cDCE. The detected low ethene suggested the potential presence of OHRB and complete reductive dechlorination with

limited activities, which would be further discussed later with the microbial community composition and functional genes response.

However, the commonly reported cDCE stalling can be observed in the current study (**Figure 2a**). Hudari et al. reported that TCE was dechlorinated to cDCE only, in both an indigenous microbial community and in bioaugmentation with *Desulfitobacterium* [22]. It was concluded that cDCE stalling could be due to the lack of obligate OHRB, capable of dechlorinating TCE to non-toxic ethene via cDCE and VC, which resulted from the lack of crucial factors (e.g., cobalamin and electron donors) for the growth and metabolism of obligate OHRB. As shown in **Table S3**, the initial TOC concentration (indicator of electron donors) in microcosm was 6.40 mg/L, which decreased to 4.12 mg/L (T-15), 4.06 mg/L (T-30), and 5.04 mg/L (T-45) after incubation in biotic treatment groups, suggesting that a portion of TOC in soil was utilized as an electron donor during the dechlorination. However, not all TOC in soil can function as effective electron donors to support the complete reductive dechlorination to ethene. As mentioned previously in Section 2.1, acetone was used as the solvent for TCE-benzene stock solutions and added to the microcosms. Although some studies have reported that acetone may undergo fermentation to provide electrons for reductive dechlorination [42, 43], the fermentation of acetone to produce molecular hydrogen required functional bacteria and mutualistic associations with hydrogenotrophic microorganisms [44, 45], suggesting that not all acetone present in the system is necessarily bioavailable or utilized. Furthermore, Li et al. found that even when exogenous electron donors are sufficiently available, additional stress factors (such as the presence of benzene in our study) may inhibit the fermentation of these electron donors and the subsequent production of bioavailable hydrogen [46]. This inhibition may result in an insufficient electron supply, thereby leading to the accumulation of intermediate products during reductive dechlorination. This finding underscores the potential of utilizing appropriate electron donors for further enhancement in the overall bioremediation, which is to be explored in our next study in the series of microcosm studies exploring the enhancement mechanisms for TEB.

Furthermore, the presence of ethene and acetylene (**Figure S1**), alongside sulfate reduction and iron reduction, supported the occurrence of abiotic degradation processes, partially contributing to the faster TCE degradation kinetics. Sulfate reduction with the generation and sulfide, also in the presence of iron reduction could form FeS, which was confirmed as a catalyzing matter for the

abiotic dichlorination of TCE [47]. It is worth noting that sulfate reduction in this study was particularly active, resulting in the increased sulfide concentrations, yet Fe(II) was not detected in the aqueous solutions in the current study (**Table S3)**, which may be due to the form of precipitated FeS. This can be further evidenced by increased iron concentrations and the significant difference value (p=0.05) between Fe(III) (0.06-0.08 mg/L) and total Fe (4.01-13.00 mg/L) also indicated the presence of insoluble Fe(II). The abiotic dechlorination of TCE by FeS was confirmed via β-elimination and hydrogenolysis, with acetylene as the main product [48]. The detections of cDCE and ethene, as well as acetylene, suggested both the biotic and abiotic dechlorination pathways for TCE [47]. However, the concentration of ethene and acetylene was much lower than that of cDCE generation, indicating that abiotic processes contributed significantly less than biotic dechlorination for TCE transformation, which was the dominant pathway for the TCE degradation.

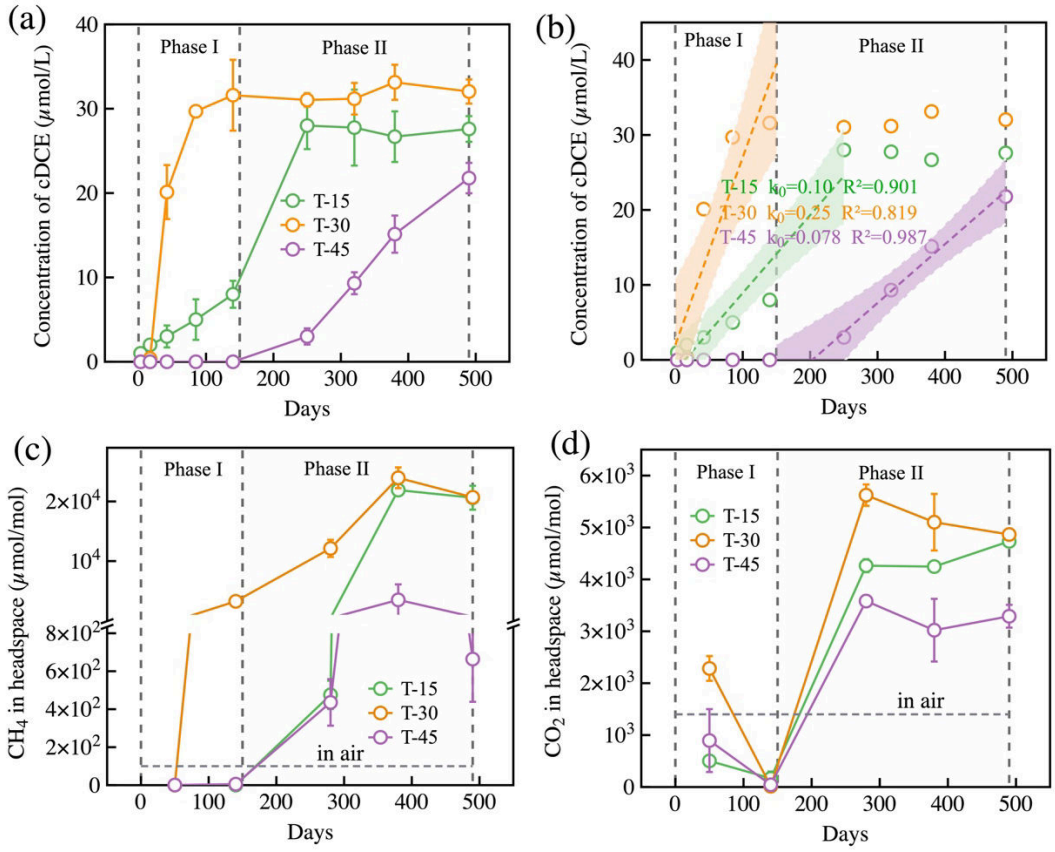

Figure 2. The formation of cDCE (a) as the primary dechlorination product of TCE and its formation kinetic (b, dash line: kinetic fitting curve, confidence = 95%) and the generation of CH_4 (c) and CO_2 (d)under thermally enhanced biodegradation for co-contaminants (Phase I: incubation

at elevated temperatures of 15 ℃, 30 ℃ and 45 ℃; Phase II: incubation at room temperature; y-axis units in c and d: volume percentage of CH_4 and CO_2 in headspace).

For benzene biodegradation, intermediates including toluene, phenol, and benzoate were identified in T-15 and T-30 after incubation (**Figure S2**), indicating their roles as by-products of benzene biodegradation. Previous researches have shown that anaerobic oxidation pathways can transform benzene into toluene, phenol and benzoate through methylation, hydroxylation and carboxylation [49, 50]. These intermediates are then further degraded to p-hydroxybenzoate [51] and ultimately mineralized to CO_2 [52]. Additionally, benzene oxidation under anaerobic conditions has been shown to occur with sulfate, nitrate, or Fe(III) serving as electron acceptors [53]. The detections of nitrite and sulfide in this study (**Table S3**) supported the existence of anaerobic benzene oxidation pathways involving sulfate reduction and limited denitrification. This finding indicated that these processes occurred concurrently, which has been reported in previous studies [33, 54].

The production of CH_4 and CO_2, indicators of microbial activities for anaerobic biodegradation for both benzene and TCE, were shown in **Figure 2c and 2d**. The generation of both CH_4 and CO_2 were the highest at T-30, followed by T-15, and T-45, consistent with the biodegradation efficiency of TCE and benzene. The production of CH_4 was significantly active and peaked at 30 ℃ in Phase I, indicating that the highest biodegradation rate of TCE occurred under the methanogenesis condition, with the highest degradation rate. However, no significant CH_4 production was observed in T-15 and T-45 in Phase I, suggesting that TCE biodegradation occurred under sulfate/Fe reducing conditions, as evidenced by the geochemistry parameters in **Table S3**. It can be concluded that the evaluated temperature of 30 ℃ induced the methanogenic condition quickly, which contributed to the highest TCE degradation and cDCE formation. After returning to ambient conditions (Phase II), the generation of CH_4 increased significantly and in T-15 it was comparable to T-30, whereas T-45 exhibited increased CH_4 production as well, although at significantly lower concentrations, which supported the continuous TCE reduction and cDCE production in T-15 and T-45 during Phase II. Moreover, the significantly elevated CH_4 concentrations in Phase II indicated that methanogens consumed a substantial portion of the available electron donors, potentially leading to competition with obligate OHRB and thereby inhibiting the further degradation of cDCE in current study [55]. Future research could focus on ensuring sufficient electron donor availability and regulating methanogenic conditions to enhance the reductive dechlorination of TCE and its

daughter products. In addition, other influencing factors, such as the toxicity on cDCE-respiring OHRB caused by benzene or reduced abundance of functional bacteria due to thermal treatment as evidenced below in the gene abundance section, can result in the incomplete dechlorination. Unlike the generation of CH_4, the generation of CO_2 decreased under all evaluated temperatures in Phase I and increased after the temperatures were adjusted to room temperature in Phase II (**Figure 2d**). The decreasing CO_2 in Phase I may be explained by hydrogenotrophic methanogenesis, in which CO_2 is consumed during CH_4 production [56]. Moreover, the highest CO_2 in both Phase 1 and Phase II were observed in T-30, consistent with the biodegradation efficiency of TCE and benzene, indicating the enhanced microbial metabolic activities during the biodegradation of TCE and benzene. Overall, this study confirmed that TCE and benzene could be degraded with native microbial community under anaerobic conditions via reductive dechlorination and oxidation, respectively. The degradation rates of TCE and benzene varied across different temperatures, implying different responses of the microbial community, particularly among contaminant-degrading bacteria and functional genes. The enhanced biodegradation mechanisms identified will be further elucidated through analyses of shifts in microbial community diversity, composition, and the dynamics of key degrading bacteria and genes in the subsequent sections.

3.2 Thermal treatment affect microbial community, functional bacteria and genes

3.2.1 Thermal effects on microbial community and co-occurrence network

The alpha diversity of the microbial community, indicated by the Chao 1 and Shannon indices, revealed significant reductions following TEB at 15 °C, 30 °C, and 45 °C when compared to the original microbial consortium (**Figure 3** and **Table S4**). This decline at 15 °C suggested that exposure to TCE, benzene, and the by-product cDCE negatively impacted microbial richness and diversity. These findings aligned with the previous study, which reported greater microbial diversity in uncontaminated samples relative to those contaminated ones [57]. Furthermore, temperature also influenced microbial alpha diversity, with a more significant decrease observed at 45 °C than 30 °C, when compared to 15 °C, consistent with the conclusion that the elevated temperatures may reduce microbial diversity [58].

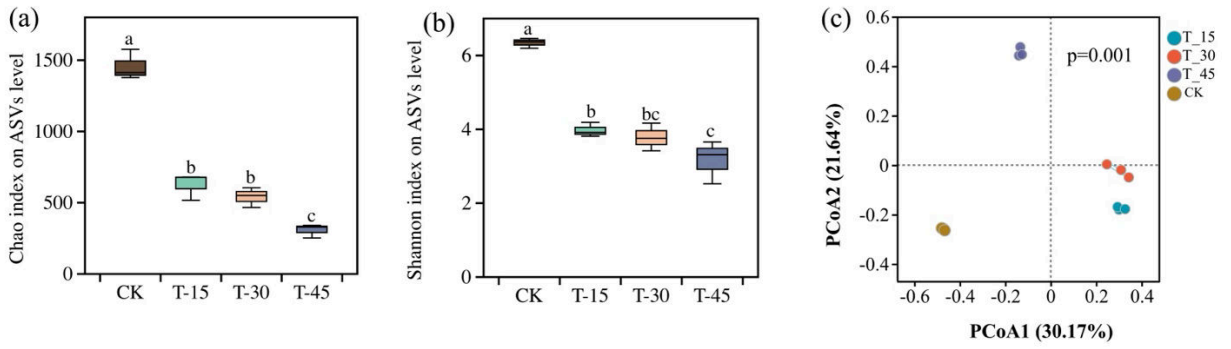

Figure 3. TEB effect on microbial community diversity **(**a and b: Alpha diversity indices. Different letters represent significant differences based on one-way ANOVA with LSD's comparisons; c: Beta diversity of principal coordinates analysis (PCoA) based on Bray-Curtis distance of ASVs level, p=0.001).

PCoA result also demonstrated that both the exposure to contaminants (T-15) and thermal treatments (T-30, T-45) had significant impacts on the microbial community, as evidenced by the separate clusters, reflecting shifts in microbial structure and compositions (**Figure 3c**). However, communities in T-15 and T-30 were closely clustered, suggesting a less significant impact compared to T-45, whereas the T-45 cluster was significantly distant from T-15 and T-30, implying substantial compositional differences at this elevated temperature. Temperature serves as a critical driver of bacterial community composition, with thermophiles prevailing at 45 °C, mesophiles at 30 °C and psychrophiles at 15 °C, leading to significant beta diversity [59].

3.2.2 Thermal effects on the biodegrading bacteria abundance

The microbial compositions at both the phylum and genus levels were shown in **Figure 4**. Following the exposure of contaminants and thermal treatment, the microbial structure exhibited similarities across temperatures, with Firmicutes predominating, followed by Chloroflexi, Acinetobacteriota, and Proteobacteria. The abundance of Firmicutes increased with temperature, consistent with previous studies that highlight their strong adaptability to thermal variations, likely due to their capacity to produce endospores that withstand extreme conditions [60]. In contrast, Proteobacteria, which was prevalent in BTEX-contaminated environments and recognized for their role in organic pollutant degradation [61], showed a marked decrease in abundance, which may contribute to the observed loss of benzene degradation in T-45. Yet in T-30, the benzene

degradation lag time was shortened, even though the significant decrease of Proteobacteria was observed, suggesting there were other functional bacteria in the consortium, which might contribute to the benzene biodegradation. Chloroflexi, which encompass essential obligate organohalide-respiring bacteria (OHRB) such as *Dehalobacter*, *Dehalogenimonas*, and *Dehalococcoides* [62], was the most abundant in T-30, consistent with the highest biotic TCE dechlorination observed. Additionally, the abundance of Actinobacteria significantly decreased in T-45, further indicating the impact of elevated temperatures on microbial community compositions and functions.

It is worth noting that it would add additional value if microbial analysis was conducted during the thermal treatment (i.e., during Phase I). Previous studies have reported that increased Firmicutes abundance was observed for benzene biodegradation when the temperature was raised from 15 °C to 30 °C and 45 °C [31]. Another study revealed that the dominating phyla switched from Proteobacteria to Firmicutes when temperature was elevated from 25 to 75 °C during the reductive dechlorination [63]. We expected that the enrichment of functional microbial bacteria (e.g., Chloroflexi) would be observed during the heating phase. More importantly, it was concluded that due to the thermal treatment at 45 °C, the inhibitive impacts on the reductive dechlorination is reversible, whereas the inhibitive effects on benzene biodegradation is non-reversible.

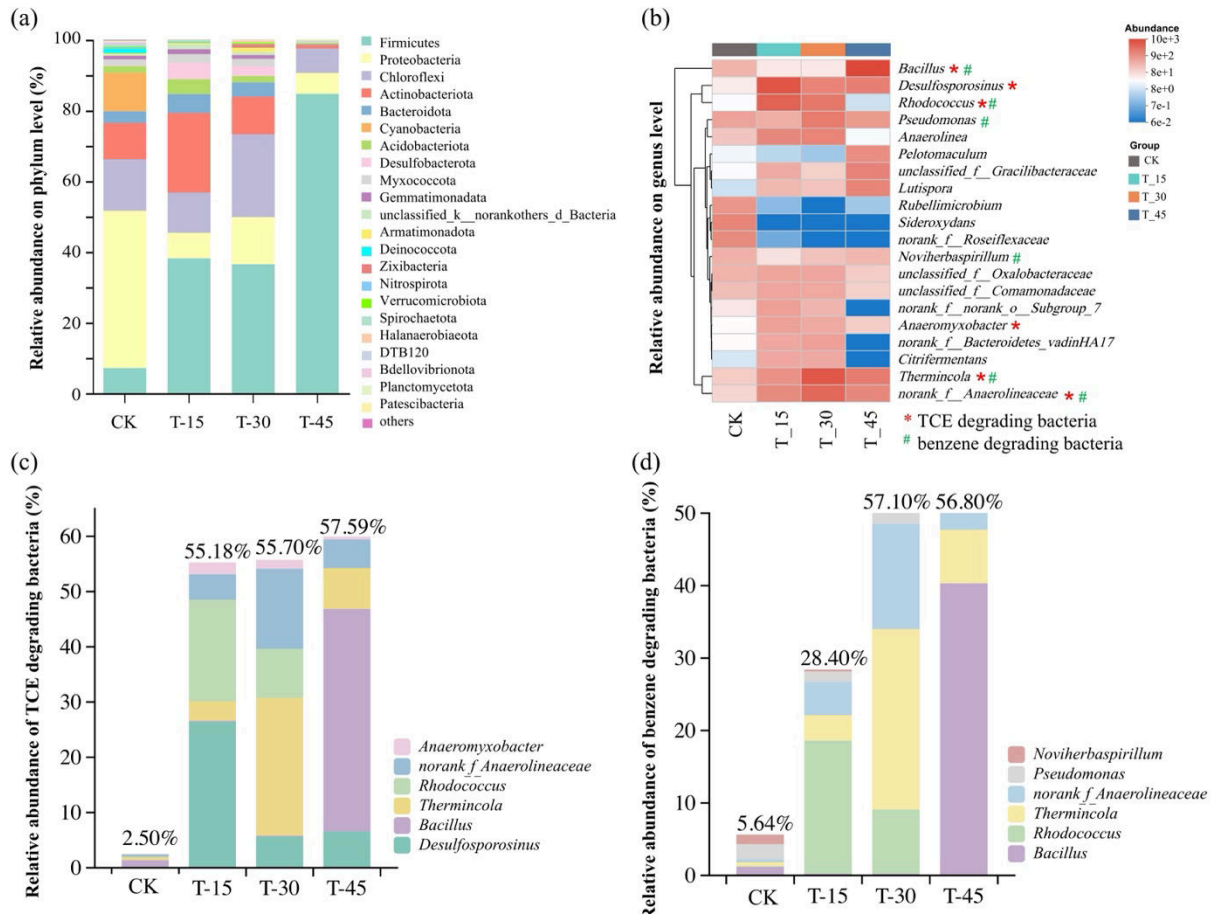

Figure 4. The microbial community compositions on phylum (a) and genus (b) levels and compositions of potential degrading bacteria for TCE (c) and benzene (d).

The thermal treatments significantly altered the composition of the top 20 abundant genera (**Figure 4b and Table S5**). *Sideroxydans* dominated the CK samples, while *Desulfosporosinus* and *Rhodococcus* were prevalent in T-15, evidencing the impacts due to the exposure to TCE and/or benzene co-contaminants. In T-30, the community shifted to include *Thermincola*, *norank_f_Anaerolineaceae* and *Pseudomonas*. *Bacillus* and *Thermincola* became the most abundant genera in T-45, which were reported having tolerance for high temperature [64, 65], indicating the thermal effects on shaping the microbial community compositions. Following the artificial co-contamination and thermal treatment, the abundance of TCE and benzene potential degrading bacteria, as listed in **Table S5** increased significantly compared to CK (**Figure 4c and 4d**). In T-15, *Desulfosporosinus* and *Rhodococcus* were most abundant genera. *Desulfosporosinus*, a sulfate-reducing bacterium linked to TCE degradation, has been shown to facilitate

dechlorination [66, 67]. *Rhodococcus* is known for its ability to degrade chlorinated solvents through reductive dechlorination and for anaerobically biodegrading benzene using hydroxylating dioxygenase [68, 69]. The increased abundance of *Desulfosporosinus* and *Rhodococcus* suggested that the presence of TCE could induce the growth of degrading bacteria, resulting in the degradation of TCE. Moreover, *Thermincola* and *norank_f_Anaerolineaceae* became the abundant TCE and benzene degrading bacteria at 30 °C. *Thermincola*, a thermophilic microorganism, has been documented to enhance dechlorination in bioelectrochemical systems [70]. Additionally, it was reported that *norank_f_Anaerolineaceae* facilitated the anaerobic reductive dechlorination and methanogenesis in flooded soils [71, 72]. Furthermore, *Pseudomonas* and *Bacillus* were also abundant in T-30, which have been widely acknowledged for degrading various contaminants, including chlorinated solvents and BTEX [73-76]. However, *Bacillus*, *Thermincola* and *Desulfosporosinus* were also dominant in T-45, with the delayed dechlorination and loss of benzene degradation, indicating their inhibited ability for contaminant degradation at much higher temperature treatment (T-45). However, it is worth noting that the dechlorination was resumed in Phase II as evidenced by the TCE concentration reduction and increase of cDCE concentration, albeit at a lower degradation rate.

Rhodococcus, norank_f_Anaerolineaceae and *Thermincola* were predominant in both T-15 and T-30, with comparable benzene degradation rates. Moreover, the loss of benzene degradation ability in T-45 was consistent with the decrease of *Rhodococcus*, indicating its potential role for participating benzene degradation. Furthermore, *Rhodococcus* growth and its metabolic activity were reported to be enhanced with temperature up to 35 °C [77, 78]. Despite the high abundance of *Bacillus* in T-45, no benzene biodegradation was observed (**Figure 1**). Previous studies reported that the optimal temperature for benzene degradation by various *Bacillus* strains is around 32 °C [28], which may cause the lost ability for benzene biodegradation at higher temperature. The findings in the current study suggested that the functional bacteria for dechlorination are more resilient when exposed to much higher temperature (45 °C), supporting the conclusion that a broader temperature range for the bioremediation of chlorinated solvents can be applied in the field than that of benzene biodegradation.

3.2.3 Thermal effects on the gene abundance

The abundances of total bacterial 16S rRNA, as well as the specific 16S rRNA genes for *Dhc* and *Dhg*, along with biodegradation functional genes for TCE (*tceA*, *vcrA*, and *bvcA*) and benzene (*tmoA* and *cat2,3*), were presented in **Figure 5** and **Table S6**. The detection of *Dhc* and *Dhg* 16S rRNA genes, as well as *tmoA* and *cat2,3* genes, confirmed their widespread presence in the original microbial community also after under different treatments. The abundance of total bacterial 16S rRNA was increased significantly in T-15, 30 and 45, while no significant difference noted among T15, T-30 and T-45, suggesting that the abundance of total genes were boosted under different treatments (**Figure 5a**). However, this increase in total bacterial abundance could be due to the thermal treatment as well as the addition of co-contaminants rising from 10^{10} to 10^{12} copies/L, compared to the original microbial consortium (CK). Additionally, the production of CH_4 (**Figure 2c**) indicated active bacterial metabolism, reflecting robust microbial growth and metabolic activity [79]. However, the observed decrease in microbial community diversity and richness under TEB, as discussed in Section 3.2.1, suggested the enrichment of thermophilic bacteria, which reduced the overall diversity while increasing the abundance.

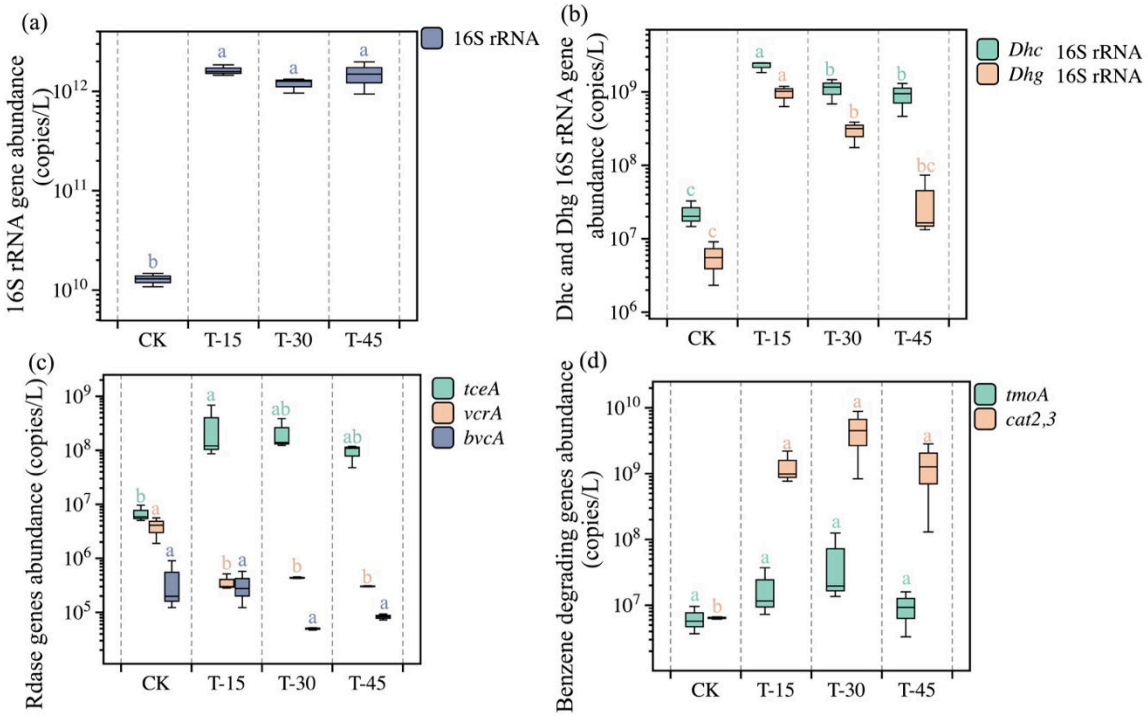

Figure 5. Gene abundance of 16S rRNA (a), *Dhc* 16S rRNA and *Dhg* 16S rRNA (b), RDase genes including *tceA*, *vcrA* and *bvcA* (c), benzene degrading functional genes including *tmoA* and *cat2,3* (d). Different letters showed significant differences ($p \leq 0.05$) among the treatments.

Similar trends were observed for the abundance of *Dhc* and *Dhg* (**Figure 5b**), with an increase of the abundance of both *Dhc* and *Dhg* 16S rRNA genes in the presence of TCE contamination compared to the CK samples, indicating their roles in TCE dechlorination. The increase in *Dhc* and *Dhg* gene abundances following TEB treatment suggests that elevated temperature may have stimulated microbial growth. Previous studies have reported that the optimal growth temperature for *Dhc* is between 15 °C and 35 °C [22], yet *Dhc* can also tolerate temperature up to 43 °C [27] and show the dechlorination activity, consistent with the observed biodegradation of TCE at 45 °C in this study. On the other hand, *Dhg* was reported with the narrower optimal growth temperature, ranging from 20 °C to 30 °C [8], suggesting that the degradation of TCE may be contributed by *Dhc* rather than *Dhg* in T-45 in this study. Moreover, it was revealed the presence of both *vcrA* and *bvcA* in **Figure 5c**. Although dechlorination stalled at cDCE, the increase in *tceA* gene copy number indicates a potential proliferation of *Dehalococcoides mccartyi* strain 195 and FL2 [80, 81], which harbor this gene. In contrast, the absence of increases in *vcrA* and *bvcA* implies that other strains (e.g., strains VS and BAV1)[82] were not similarly enriched. This discrepancy may reflect physiological differences among *Dhc* strains and their temperature responses. Moreover, the abundance of *tceA* followed the same trend of *Dhc*, suggesting that *tceA* may be responsible for the TCE degradation rather than *vcrA* and *bvcA* genes in this study. However, it was reported that VC-respiring OHRB were more easily perturbed by unfavorable environmental conditions (evaluated temperature, the potential insufficiency of hydrogen and the presence of benzene in this study) than TCE-respiring OHRB [46], which may result to the negative response of *vcrA* and *bvcA* with reduced abundance compared to the increased *tceA* during TEB process in this study, leading to cDCE stalling.

In addition, the abundance of *tmoA* and *cat2,3* both increased in TEB groups, compared to CK, implying the presence of benzene and thermal treatment could induce the enrichment of degrading genes. Besides, the highest abundances of *tmoA* and *cat2,3* were observed in T-30 with high benzene degradation rate, suggesting the contributions of *tmoA* and *cat2,3* to the enhanced biodegradation of benzene at 30 °C (**Figure 5d**). The positive correlation between *tmoA* abundance

and the benzene degradation rate further underscored its critical role in the initial oxidation of benzene (**Figure S3**). Moreover, the observation of toluene, phenol, benzoate in Section 3.1.2 confirmed the oxidation pathway of benzene in this study, validating the participation of *tmoA* and *cat2,3* in these processes. Although the *tmoA* gene is typically associated with aerobic toluene monooxygenase activity that requires molecular oxygen, a previous study reported that anaerobic oxidation of benzene can proceed via mechanisms that utilize oxygen generated internally from water with the evidence of formation of ^{18}O-labelled phenol from ^{18}O-labelled water [83], enabling the activity of oxygen-dependent enzymes such as *tmoA*. The results in the current study showed notable increase in *tmoA* gene abundance under anaerobic conditions (**Figure 5d**), along with a previous study [84],suggesting that *tmoA* may utilize oxygen originated from other compounds (e.g., sulfate in this study with decreased concentration and the formation of sulfide, **Table S3**) and play a role in benzene degradation under anaerobic conditions. However, the exact functional role of *tmoA* in anaerobic benzene degradation remains unclear. Further research using genomic and transcriptomic approaches is needed to identify the key genes and mechanisms involved in anaerobic oxidation processes.

Likely with *tmoA*, the abundance of *cat2,3* also increased after the microbial community being exposed to co-contaminants and different temperature and at its lowest at 45°C. Despite the abundance of *cat2,3* being increased significantly at 45 °C compared to the original microbial consortium, no benzene degradation was observed. Moreover, *tmoA* was responsible for the initial attack to benzene ring and then the intermediate could be served as substrate for enzyme encoded by *cat2,3*. The decrease of *tmoA* at 45 °C resulted in the restriction to initial process of benzene degradation, causing the insufficient substrate for *cat2,3*. Under this condition, *cat2,3* didn't lead to benzene degradation, even with increased abundance in T-45. It has been reported that higher temperatures can result in damage to the structure of enzymes, thus reducing their contaminant degradation capacity [85], which may cause the lost catalytic activity of enzyme encoded by *tmoA* and inhibition of benzene biodegradation at 45 °C.

3.3 Mechanisms for thermally enhanced biodegradation

A comprehensive scheme illustrating the key TEB mechanisms on TCE and benzene co-contaminants was proposed in **Figure 6**. Four distinctive degradation processes were identified for TCE and benzene under co-contamination conditions involving the indigenous microbial

community. The anaerobic oxidation of benzene was proposed via the methylation, hydroxylation and carboxylation pathways, supported by the identification of by-products such as toluene, phenol, benzoate, as well as CO_2 and CH_4. Notably, the primary biotic reductive dechlorination of TCE to cDCE was observed under anaerobic conditions in groundwater. Additionally, the biogeochemical processes of nitrate, sulfate and iron reductions accompanying the TCE and benzene degradation were proved to be actively participating in the microbial activities of the native consortium. Furthermore, the abiotic degradation of TCE facilitated by FeS, evidenced by the detections of acetylene and ethene, albeit at much lower concentrations, accounted for a minor contribution to the observed TCE degradation.

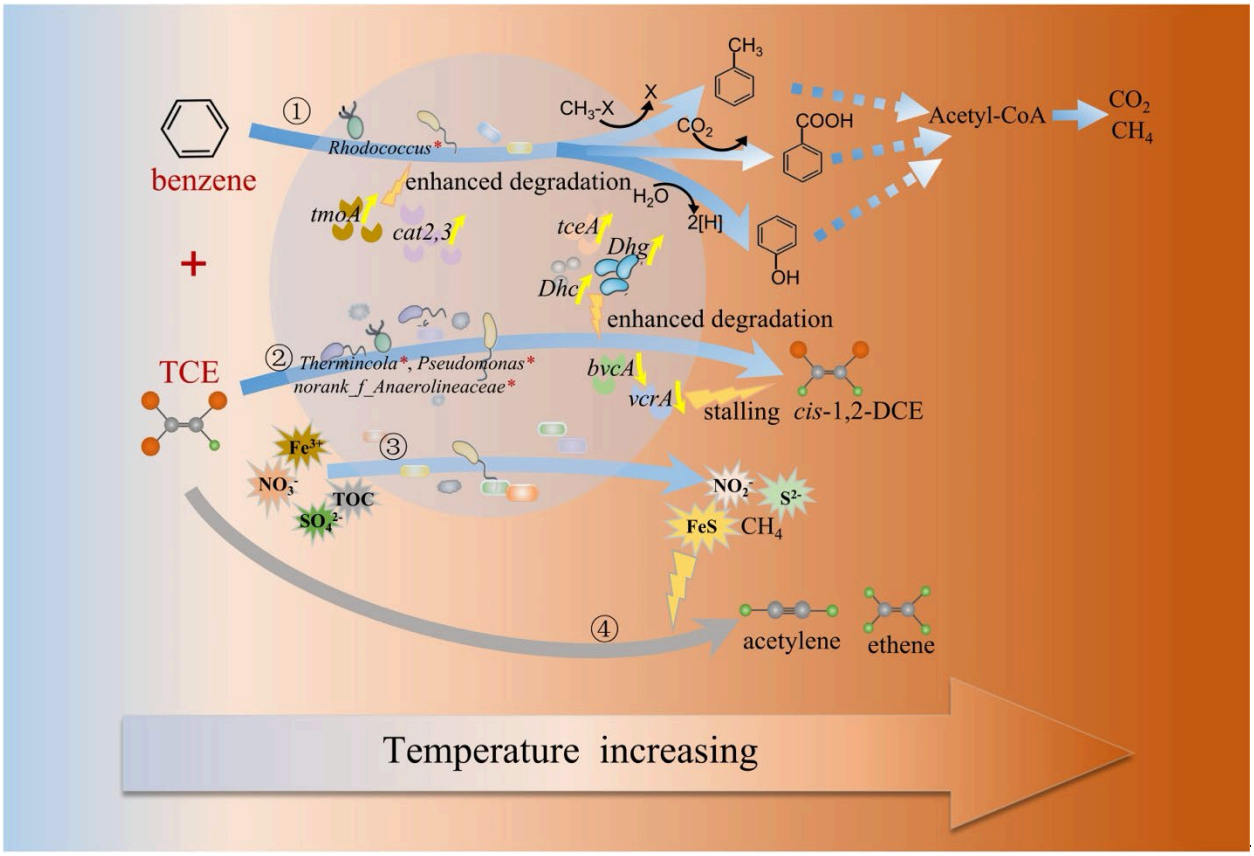

Figure 6. Mechanisms of thermally enhanced biodegradation of co-contamination of TCE and benzene with indigenous community (① anaerobic oxidation of benzene, including initial attack of benzene ring via methylation and hydroxylation and then carboxylation, finally to CO_2 and CH_4, ② biotic reductive dechlorination of TCE to cDCE, ③ sulfate reduction, denitrification, iron reduction and methanogenesis, ④ abiotic dechlorination of TCE).

The mechanisms of TEB that led to enhanced biodegradation rates of both TCE and benzene can be summarized as follows: (1) the thermal treatment effectively enriched bacterial populations capable of degrading TCE and benzene, resulting in higher overall abundances compared to the pre-treatment community. In the current study, *Thermincola*, *norank_f_Anaerolineaceae*, and *Pseudomonas* were the potential key players in TCE degradation, while *Rhodococcus* predominantly contributed to benzene degradation; (2) TCE was primarily dechlorinated to cDCE during thermal treatment, as evidenced by an increase in *Dhc* and *Dhg* gene copies from 10^7 to 10^9 copies/L, and an increase in *tceA* gene abundance from 10^6 to 10^8 copies/L. However, the stalling of cDCE degradation was likely due to the insufficiency of suitable electron donors and negative response of *vcrA* and *bvcA* genes to elevated temperatures. The *tceA* gene was particularly influential in the dechlorination of TCE to cDCE. Similarly, the abundances of benzene biodegradation functional genes, *tmoA* and *cat2,3*, increased with temperature, indicating that *tmoA* is the key functional gene responsible for the initial attack in benzene degradation; (3) TEB process involved integrated biological activities, including limited denitrification, sulfate reduction, Fe(III) reduction and methanogenesis processes, which facilitated the dechlorination of TCE and supplied electron acceptors during the anaerobic oxidation of benzene. Furthermore, the formation of FeS from sulfate and iron reduction supported the abiotic degradation of TCE, with generation of acetylene and ethene. This can be crucial for the application of TEB at contaminated sites. These findings indicated that TEB at evaluated temperatures has great potential for the effective removal of TCE and benzene co-contaminants.

4. Conclusions

This study provided novel insights into the TEB of TCE and benzene co-contaminants, elucidating the complex interactions among temperature, microbial communities, and biodegradation pathways. It highlighted the crucial roles of indigenous microbial populations in facilitating TCE reductive dechlorination and anaerobic oxidation of benzene, identifying thedominant functional genera, including *Thermincola*, *norank_f_Anaerolineaceae*, *Pseudomonas* and *Rhodococcus* as significant contributors to these processes. Moreover, the findings further emphasized the impact of thermal treatment on microbial dynamics, showing that evaluated temperature at 30 °C not only enrich populations capable of degrading contaminants but also influence the essential functional genes. The increase in *Dhc* and *Dhg* gene abundance from 10^7 to 10^9 copies/L, *tceA* gene from 10^6

to 10^8 copies/L, *tmoA* and *cat2,3* from 10^6 to 10^7 and 10^9 copies/L, respectively, were observed at, facilitating the biodegradation of TCE and benzene. In addition, the study elucidated the abundance and correlation of functional genes, including *tceA*, *tmoA*, and *cat2,3*, with the extent of biodegradation of benzene and TCE, laying the groundwork for developing molecular tools to monitor and optimise bioremediation efforts. Nevertheless, the reduced *vcrA* and *bvcA* under TEB and insufficient effective electron donor may account for cDCE stalling.

Therefore, to enhance the effectiveness of TEB for complete dechlorination and removal of benzene in practical applications, additional strategies such as biostimulants amendment (e.g., suitable electron donors, electron acceptors or nutrients) and integration with soil vapor extraction to remove free-phase or volatile compounds should be considered. Furthermore, the integration of multiple biological processes, such as methanogenesis and sulfate reduction during TEB in the presence of soil, further highlighted the complexity of microbial interactions at contaminated sites, suggesting that leveraging these interactions could enhance remediation effectiveness. Overall, this study advanced our understanding of TEB mechanisms in co-contaminated environments, providing a robust framework for future research focused on improving remediation technologies for chlorinated and aromatic hydrocarbon pollutants, ultimately contributing to more effective groundwater management.

Author contributions

Zhuanxia Zhang: Writing original draft, Methodology, Conceptualization. **Mukhtiar Ali**: Validation, Methodology, Conceptualization. **Qing Wang**: Methodology, Conceptualization. **Frederic Coulon**: Writing review & editing. **Xin Song**: Writing review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability statement: All data generated and analysed during this study are included in this article and its supplementary information files.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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