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Biological effect of temperature changes on microbial communities and groundwater contaminations

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Sworn declaration

Hereby I state,

- that apart from the supervisors' guidance the content and design of the thesis is all my own work;
- that the thesis has not already been submitted either partially or wholly as part of a doctoral degree to another examining body;
- that parts (chapters 2 to 6) of this thesis have been published in academic journals;
- that the thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation and the Helmholtz Centre for Environmental Research – UFZ;
- that no academic degree has ever been withdrawn.

Paris, 17 April 2026

Mohammad Sufian Bin Hudari



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Dissertation summary

Aquifer Thermal Energy Storage (ATES) provides a sustainable means of balancing seasonal heating and cooling demand but introduces temperature fluctuations that can influence subsurface microbial processes essential for groundwater quality. This thesis summarizes investigations of how temperature governs anaerobic microbial activity, community composition, and contaminant transformation in aquifer environments.

Microcosm experiments combining stable isotope analysis (Gas chromatography – isotope ratio mass spectrophotometer (GC–IRMS)) and molecular community profiling were used to quantify acetate and toluene mineralisation under sulfate-reducing conditions and to identify the key organisms involved. Degradation of trichloroethene (TCE) was monitored by gas chromatography with flame ionisation detection (Gas chromatography – flame ionization detector (GC–FID)) under methanogenic and reductive dechlorination conditions. Depending on the extent of dechlorination, intermediate products were formed.

Moderate warming (12–25 °C) enhanced sulfate reduction and carbon mineralisation, whereas heating above 38–45 °C inhibited activity. Active sulfate-reducing cultures persisted at 45 °C, indicating limited thermotolerance and partial recovery after heat exposure. In contrast, complete TCE dechlorination occurred up to 20 °C, became incomplete at 30–40 °C, and was inhibited at higher temperatures.

Community analyses revealed temperature-driven compositional shifts: functional redundancy and thermal memory supported resilience within the mesophilic range, yet prolonged heating reduced diversity, extended lag phases, and favoured moderately thermotolerant taxa with incomplete functional recovery.

These results define microbial thresholds relevant to ATES operation—approximately 45 °C for mineralisation of toluene and acetate as model organic compounds at sulfate-reducing conditions and $\leqslant$20 °C for complete TCE dechlorination—and underscore the importance of site-specific, microbially informed temperature management to enable sustainable, low-carbon energy storage without compromising groundwater ecosystem integrity.

Zusammenfassung

Aquifer Thermal Energy Storage (ATES) stellt einen nachhaltigen Ansatz zur Deckung des saisonalen Wärme- und Kältebedarfs dar, führt jedoch zu Temperaturschwankungen, die mikrobiologische Prozesse im Untergrund und damit die Grundwasserqualität beeinflussen können. In dieser Dissertation wurde untersucht, wie die Temperatur die anaerobe mikrobielle Aktivität, die Zusammensetzung mikrobieller Gemeinschaften und die Umwandlung von Schadstoffen in Aquiferen steuert.

Mikrokosmenexperimente, kombiniert mit stabiler Isotopenanalyse (GC–IRMS) und der Charakterisierung mikrobieller Gemeinschaften, wurden eingesetzt, um die Mineralisierung von Acetat und Toluol unter sulfatreduzierenden Bedingungen zu quantifizieren und die Schlüsselorganismen zu identifizieren. Der Abbau von Trichlorethen (TCE) wurde mittels Gaschromatographie mit Flammenionisationsdetektion (GC–FID) unter methanogenen und reduktiven Dechlorierungsbedingungen untersucht, wobei je nach Ausmaß der Dechlorierung unterschiedliche Zwischenprodukte gebildet wurden. Moderate Erwärmung (12–25 °C) förderte die Sulfatreduktion und den Kohlenstoffumsatz, während Temperaturen über 38–45 °C die Aktivität hemmten. Sulfatreduzierende Kulturen blieben bis 45 °C metabolisch aktiv und zeigten eine begrenzte Thermotoleranz sowie eine teilweise Erholung nach Hitzeeinwirkung, wohingegen eine vollständige TCE-Dechlorierung nur bis 20 °C nachgewiesen wurde, zwischen 30–40 °C unvollständig war und bei höheren Temperaturen gehemmt wurde.

Die Analysen der mikrobiellen Gemeinschaften zeigten temperaturabhängige Verschiebungen in der Zusammensetzung: Funktionelle Redundanz und thermische „Erinnerung“ förderten die Resilienz im mesophilen Bereich, während sich bei längerer Erwärmung die Diversität verringerte, Lagphasen verlängerten und mäßig thermotolerante Taxa anreicherten – jedoch ohne vollständige Wiederherstellung der Funktion des Mikrobioms.

Diese Ergebnisse definieren mikrobiologische Temperaturschwellen, die für den ATES-Betrieb relevant sind – etwa 45 °C für die sulfatreduzierende Mineralisierung von Toluol und Acetat sowie $\leqslant$20 °C für die vollständige TCE-Dechlorierung – und unterstreichen die Bedeutung eines standortspezifischen, mikrobiologisch fundierten Temperaturmanage-

ments, um eine nachhaltige, CO_2-arme Energiespeicherung zu ermöglichen, ohne die Integrität des Grundwasserökosystems zu stören.

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List of abbreviations and acronyms

16S rRNA 16 Svedberg ribosomal ribonucleic acid

ATES Aquifer Thermal Energy Storage

BGS below ground surface

BTEX benzene-toluene-ethylbenzene-xylenes

DCE dichloroethene

DMPD N,N-dimethyl-1,4,-diphenylenediammonium dichloride

DNA deoxyribonucleic acid

EU European Union

GC–FID Gas chromatography – flame ionization detector

GC–IRMS Gas chromatography – isotope ratio mass spectrophotometer

HT-ATES high temperature aquifer thermal energy storage

LT-ATES low temperature aquifer thermal energy storage

PCE perchloroethene

PCR polymerase chain reaction

SIP stable isotope probing

TCE trichloroethene

U.S. EPA United States Environmental Protection Agency

VC vinyl chloride

VPDB Vienna Pee Dee Belemite

Chapter 1.

Introduction

> Heat energy of uniform temperature [is] the ultimate fate of all energy. The power of sunlight and coal, electric power, water power, winds and tides do the work of the world, and in the end all unite to hasten the merry molecular dance
>
> Frederick Soddy

In 1896, Arrhenius linked atmospheric CO_2 to climate warming, and in 1938, Callender associated rising temperatures with fossil fuel use—the "Callender Effect" (Anderson et al., 2016; Callendar, 1938). Despite the 2016 Paris Agreement (United Nations Framework Convention on Climate Change, 2015), 2024 was reported as the warmest year on record, likely exceeding 1.5 °C above pre-industrial levels (National Oceanic and Atmospheric Administration, 2025). While European Union (EU) energy-sector CO_2 emissions fell by approximately 11% in 2024, global energy-related emissions still increased by 0.8% (International Energy Agency, 2025). These trends underscore the urgent need for low-carbon heating and cooling solutions.

Aquifer Thermal Energy Storage (ATES) is a promising option for the heating and cooling sector, which has historically been highly dependent on fossil fuels. First applied for thermal storage in China, ATES later demonstrated bioremediation potential in the Netherlands, which now leads in both research and implementation. Germany and France, despite favorable hydrogeological conditions, continue to lag behind (Fleuchaus et al., 2018). Persistent skepticism regarding subsurface heating and bioremediation performance (Fleuchaus et al., 2018) underscores the need for further investigation.

Scope. In this thesis, the influence of temperature on the biotransformation of toluene

(primary) and chlorinated ethenes (secondary)—common groundwater contaminants in Europe—was investigated in the context of ATES and natural attenuation.

1.1. Heating and cooling: a hard-to-abate sector

Heating and cooling account for nearly half of global final energy use. As of 2020, approximately 60% of this demand was supplied by fossil fuels, around 30% by biomass, and the remainder by other renewables (International Renewable Energy Agency and International Energy Agency and REN21, 2020). In Europe, the share is about 51%, still predominantly fossil-based, although the contribution of renewables has increased to 18–23% in recent years (European Commission, 2016). Global demand for heating—and particularly for cooling—is projected to increase substantially by 2050, driven by population growth, urbanisation, and rising ambient temperatures (European Union, 2023). In line with European climate targets, current policy prioritises energy efficiency and the decarbonisation of the heating and cooling sector. While low-temperature industrial and residential heating demands are increasingly electrified, high-temperature applications remain difficult to decarbonise due to their high energy intensity and reliance on combustion-based technologies (International Renewable Energy Agency and International Energy Agency and REN21, 2020). Seasonal thermal energy storage technologies, such as ATES, offer a promising pathway to reduce fossil fuel dependence and enhance renewable energy utilisation throughout the year.

1.2. Aquifer thermal energy storage: principles and significance

Concept and rationale. ATES is an open-loop geothermal technology that stores and recovers heat in aquifers to balance seasonal energy demand. It functions primarily as an energy *conservation or efficiency* measure, enabling the reuse of stored thermal energy rather than its dissipation, thereby increasing the renewable share in heating and cooling and reducing CO_2 emissions by several thousand tonnes per year (Fleuchaus et al., 2018). Declining investment and operational costs have made ATES competitive with other subsurface thermal storage systems, such as Borehole Thermal Energy Storage (BTES), and in some contexts even with hydrogen storage caverns (HEATSTORE, 2021; Herrmann et al., 2026). ATES can also be integrated with solar and wind energy systems or used to

recover and store excess industrial waste heat, thereby enhancing overall system efficiency and reducing energy waste.

Operating principle. Most ATES installations are bidirectional doublet systems comprising paired "warm" and "cold" wells operated alternately between summer and winter. Groundwater is circulated between the wells to provide cooling in summer and heating in winter, with the wells spaced sufficiently apart to minimise thermal interference and maintain seasonal thermal balance (Dickinson et al., 2009; Jackson et al., 2024; Stemmle et al., 2022). The general operation of these systems is illustrated in Figure 1.1. Single-borehole ATES (SB-ATES) designs use vertically separated well screens within a single borehole to reduce hydraulic short-circuiting and thermal loss (Fleuchaus et al., 2018).

Regional climatic conditions strongly influence ATES feasibility and operational efficiency, as they determine the relative demand for heating and cooling. Table 1.1 summarises the monthly average temperature, heating degree days (HDDs), and cooling degree days (CDDs) for Germany in 2024, providing a quantitative context for evaluating potential seasonal storage performance.

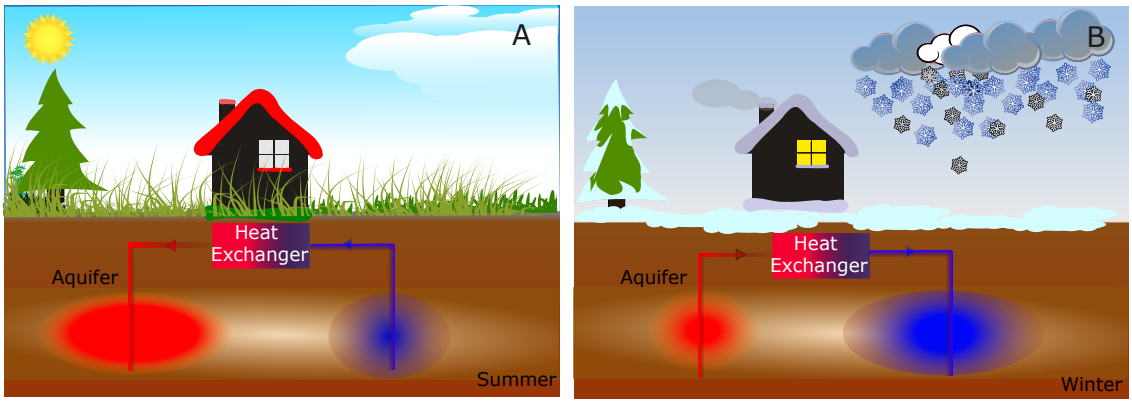


Figure 1.1.: Simplified schematic illustrating the operational principle of Aquifer Thermal Energy Storage (ATES). (A) Summer operation (cooling mode), where excess building heat is transferred to groundwater via a heat exchanger and stored in the warm well. (B) Winter operation (heating mode), where stored thermal energy is recovered from the aquifer to supply building heat. The diagram highlights the seasonal bidirectional heat exchange between the subsurface and the built environment, illustrating the core ATES cycle.

Deployment & barriers. Since 2018, approximately 2,800 ATES systems have been in operation worldwide, about 85% of which are located in Europe—predominantly in the Netherlands (Fleuchaus et al., 2018). The vast majority (∼99%) are low-temperature systems (low temperature aquifer thermal energy storage (LT-ATES); <25 °C), while high-temperature systems (high temperature aquifer thermal energy storage (HT-ATES))

Table 1.1.: Monthly average temperature and heating and cooling degree days (HDD and CDD) in Germany for 2024. HDD and CDD are cumulative temperature indices (expressed in degree days, °C·d) that quantify the demand for space heating or cooling. They are calculated as the sum of daily mean temperature differences below (HDD) or above (CDD) a base temperature (typically 15 °C). Higher HDD values indicate colder conditions and increased heating demand, whereas higher CDD values indicate warmer conditions and increased cooling demand. Temperature data are from Statista (statista.com), and HDD/CDD data are from Eurostat (ec.europa.eu).

Month	**Average Temp (°C)**	**HDD**	**CDD**
Jan	1.5	503.32	0.00
Feb	6.6	319.99	0.00
Mar	7.5	310.45	0.00
Apr	10.0	218.87	0.00
May	14.9	63.36	0.00
Jun	16.8	40.36	2.81
Jul	18.9	8.33	3.02
Aug	20.0	1.18	9.06
Sep	15.3	74.15	4.41
Oct	11.0	195.20	0.00
Nov	4.5	375.35	0.00
Dec	2.2	458.19	0.00

remain relatively uncommon. Nevertheless, HT-ATES offers greater storage capacities and higher thermal efficiencies at comparable flow rates (Daniilidis et al., 2022; Drijver et al., 2012).

Regulatory frameworks vary among countries, defining either absolute operational temperature limits or permissible temperature differences (ΔT) in groundwater (Bonte et al., 2011a; Haehnlein et al., 2010; Hähnlein et al., 2013; Rudolph et al., 2018). In Germany, current thresholds are 20 °C for heating and 5 °C for cooling at shallow depths (Fleuchaus et al., 2018; Haehnlein et al., 2010). Despite these relatively conservative regulations, the national potential is considerable: approximately 54% of Germany's surface area has been classified as "well" or "very well" suited for ATES deployment by 2050 (Stemmle et al., 2022). Figure 1.2 illustrates this suitability potential (A) and the current state of ATES research and implementation in Germany (B). Only a few operational systems currently exist (e.g., Bonn and Rostock), with additional sites under development, such as in Karlsruhe (Fleuchaus et al., 2021; Schüppler et al., 2019; Stemmle et al., 2025a).

Socio-economic barriers—including limited public awareness, complex permitting procedures, and high initial investment costs—continue to constrain broader implementation despite the proven technical maturity of the technology (Bonte et al., 2011a; Fleuchaus et al., 2018; Haehnlein et al., 2010; Stemmle et al., 2025b).

Contamination in urban aquifers

Urban and industrial activities have introduced numerous organic contaminants—most notably benzene, toluene, ethylbenzene, and xylenes (BTEX) and chlorinated hydrocarbons—into groundwater systems across Europe (Huang et al., 2014; Yu et al., 2022). The U.S. Agency for Toxic Substances and Disease Registry (ATSDR) lists benzene-toluene-ethylbenzene-xylenes (BTEX) compounds among its priority pollutants due to their frequency, toxicity, and potential for human exposure (Agency for Toxic Substances and Disease Registry (ATSDR), 2024). Accordingly, the U.S. Environmental Protection Agency (USEPA) has set maximum contaminant levels (MCLs) of 5, 1000, 700, and 10,000 $\mu g\,L^{-1}$ for benzene, toluene, ethylbenzene, and xylenes, respectively (United States Environmental Protection Agency (EPA), 2020). The physicochemical properties and regulatory limits of these and related chlorinated ethenes are summarised in Table 1.2.

In Europe, BTEX and chlorinated hydrocarbons such as perchloroethene (PCE) and TCE were estimated in 2013 to affect approximately 18% and 24% of contaminated soils and groundwater, respectively (Panagos et al., 2013). However, more recent continent-wide assessments are scarce, and comprehensive data on the current extent of pollution remain

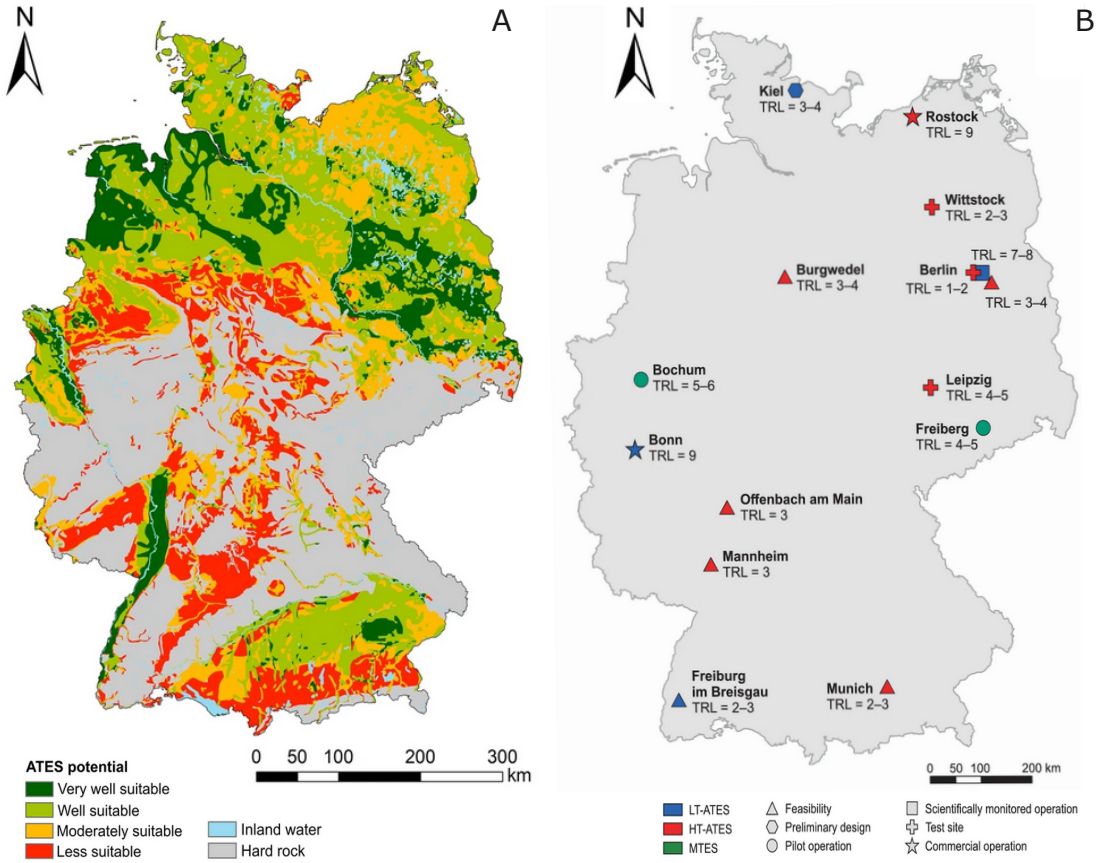


Figure 1.2.: (A) ATES suitability potential in Germany, adapted from Stemmle et al. (2022). (B) Current ATES research and implementation status in Germany, adapted from Stemmle, Arab, et al. (2025a).

limited (Arena for Journalism in Europe and Datadista, 2025).

Groundwater provides nearly 65% of the public drinking-water supply in the European Union (European Environment Agency, 2022), making contamination of aquifers beneath densely populated or industrialised areas a critical environmental and public-health concern. To safeguard this resource, EU policy now promotes the zero-pollution hierarchy, which mandates remediation when prevention or minimisation of contamination is not feasible (European Commission, 2021; European Commission, Directorate-General for Research and Innovation, 2021). Against this backdrop of widespread groundwater contamination and policy-driven remediation goals, bioremediation—particularly natural attenuation—has emerged as a sustainable and cost-effective approach to restore aquifer quality.

Table 1.2.: Physicochemical properties and U.S. EPA maximum contaminant levels (MCLs) for selected BTEX compounds and chlorinated ethenes. Solubility and vapour pressure values were compiled from peer-reviewed sources and chemical databases including ATSDR (Agency for Toxic Substances and Disease Registry (ATSDR), 2007), EPA (U.S. Environmental Protection Agency, 2011), Sigma-Aldrich (Sigma-Aldrich, 2011), and NIST WebBook (National Institute of Standards and Technology (NIST), 2024). MCLs follow U.S. EPA national primary drinking water standards (https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations). German drinking water limits are based on the Trinkwasserverordnung (2023). *Entries marked with "–" indicate that no specific German limit is defined.*

Compound	**Solubility** (mg/L)	**Density** (g/cm^3)	**Vapour Pressure** (mmHg @25°C)	**EPA MCL** (μg/L)	**German limit** (μg/L)
Benzene	1790	0.88	95	5	1
Toluene	515	0.87	28.4	1000	–
Ethylbenzene	152	0.87	9.5	700	–
Xylenes	106	0.86	6.6	10000	–
TCE	1100	1.46	58	5	10
cis-DCE	3500	1.28	200	70	–
Vinyl chloride	2700	0.91	2600	2	0.5

1.3. Anaerobic transformation in aquifers

In most subsurface environments, oxygen is rapidly consumed through microbial respiration and chemical oxidation of reduced minerals, resulting in predominantly anoxic

Table 1.3.: Approximate standard Gibbs free energy changes ($\Delta G^{\circ\prime}$) for toluene oxidation under aerobic and anaerobic respiration, and in syntrophic associations. Values are given per mole of toluene (C_7H_8) unless stated otherwise. In this table, "complete" sulfate reduction refers to full oxidation of toluene to bicarbonate, whereas "incomplete" sulfate reduction denotes partial oxidation leading to acetate formation. Adapted from Lueders (2017), Weelink et al. (2010), and Widdel et al. (2010) .

Electron acceptor (oxidised/reduced)	**Stoichiometry**	$\Delta G^{\circ\prime}$ **(kJ mol^{-1} C_7H_8)**
O_2/H_2O	$C_7H_8 + 9\,O_2 + 3\,H_2O \rightarrow 7\,HCO_3^- + 7\,H^+$	-3790
NO_3^-/N_2	$5\,C_7H_8 + 36\,NO_3^- + H^+ \rightarrow 35\,HCO_3^- + 18\,N_2 + 3\,H_2O$	-3555
$Fe(OH)_3/FeCO_3$	$C_7H_8 + 36\,Fe(OH)_3 + 29\,HCO_3^- + 29\,H^+ \rightarrow 36\,FeCO_3 + 87\,H_2O$	-1497
SO_4^{2-}/HS^- (complete)	$2\,C_7H_8 + 9\,SO_4^{2-} + 6\,H_2O \rightarrow 14\,HCO_3^- + 9\,HS^- + 5\,H^+$	-203
	per mol SO_4^{2-}	(-45)
SO_4^{2-}/HS^- (incomplete)	$2\,C_7H_8 + 3\,SO_4^{2-} + 6\,H_2O \rightarrow 6\,CH_3COO^- + 2\,HCO_3^- + 3\,HS^- + 5\,H^+$	-61
	per mol SO_4^{2-}	(-41)
CO_2/CH_4 (sum)	$2\,C_7H_8 + 15\,H_2O \rightarrow 9\,CH_4 + 5\,HCO_3^- + 5\,H^+$	-130
Fermentation	$2\,C_7H_8 + 18\,H_2O \rightarrow 6\,CH_3COO^- + 2\,HCO_3^- + 8\,H^+ + 12\,H_2$	$+166$
Hydrogenotrophic methanogenesis	$12\,H_2 + 3\,HCO_3^- + 3\,H^+ \rightarrow 3\,CH_4 + 9\,H_2O$	-203
Acetotrophic methanogenesis	$6\,CH_3COO^- + 6\,H_2O \rightarrow 6\,CH_4 + 6\,HCO_3^-$	-93

conditions (Farhadian et al., 2008; Lueders, 2017; Meckenstock et al., 2015; Miao et al., 2012; Rittmann et al., 2000; US Environmental Protection Agency, 1999; Weelink et al., 2010). Under these conditions, microorganisms rely on alternative terminal electron acceptors—such as nitrate, ferric iron, sulfate, and carbon dioxide—to sustain energy metabolism. This sequential use of electron acceptors establishes characteristic redox zonation within contaminated aquifers, typically progressing from oxic zones at plume margins to strongly reducing zones in the core. The spatial distribution of these anaerobic processes within a hydrocarbon plume is illustrated in Figure 1.3, based on the plume fringe concept (Meckenstock et al., 2015).

As illustrated, overlapping redox processes at the plume fringes create narrow but reactive transition zones where contaminant degradation is often most active. These mixed redox interfaces are key to sustaining natural attenuation, as they facilitate concurrent use of multiple electron acceptors and enhance microbial diversity within the plume.

The thermodynamic hierarchy of microbial respiratory processes is governed by the energy yields associated with their respective redox reactions. Aerobic respiration provides the highest energy gain, whereas sulfate reduction, syntrophic oxidation, and methanogenesis yield substantially less. The Gibbs free energies for toluene oxidation under different respiratory conditions (Table 1.3) illustrate this hierarchy: oxidation coupled to oxygen or nitrate reduction provides the largest energy yields, followed by iron(III) and sulfate reduction, while syntrophic and methanogenic pathways offer only marginal or even positive $\Delta G^{\circ\prime}$ values.

Within these redox gradients, plume fringes often exhibit the highest biodegradation rates because electron donors (e.g., organic contaminants) and electron acceptors co-occur, creating “hot spots” of microbial activity (Anneser et al., 2008; Meckenstock et al., 2015; Tischer et al., 2013; Tuxen et al., 2006; van Breukelen & Griffioen, 2004). In many contaminated aquifers, management and regulatory frameworks favour sulfate-reducing conditions over nitrate reduction due to nitrate’s potential eutrophication risk and regulatory constraints (Cunningham et al., 2001; Reinhard et al., 1996). However, sulfate reduction may also lead to sulfide accumulation, which can pose secondary geochemical challenges (Canfield et al., 1992).

Intrinsic biodegradation of BTEX under sulfate-reducing conditions is well documented in field studies across diverse aquifers, confirming that anaerobic transformation can be a major natural attenuation process for hydrocarbons (Davis et al., 1999; Gieg et al., 1999).

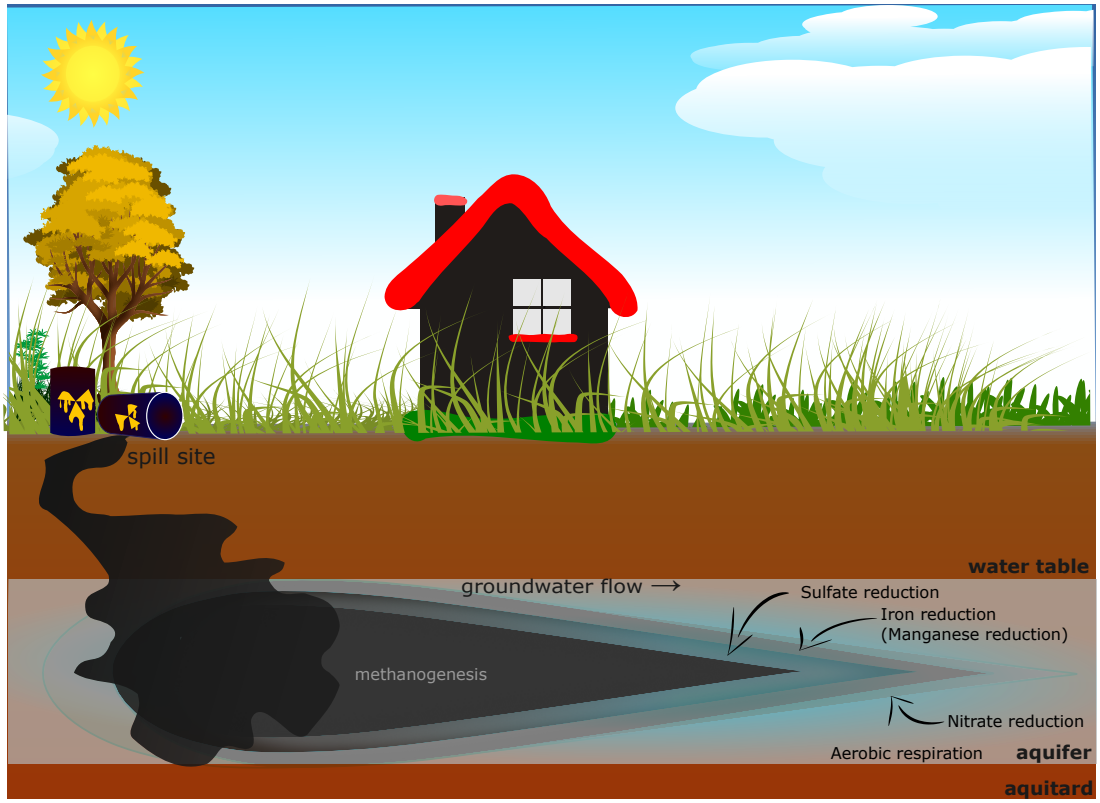


Figure 1.3.: Conceptual illustration of redox zonation in a groundwater contaminant plume. Electron acceptors are consumed sequentially ($O_2 \rightarrow NO_3^- \rightarrow$ Fe(III) $\rightarrow SO_4^{2-} \rightarrow CO_2$). Highest biodegradation rates often occur at plume fringes where donors and acceptors overlap. Schematic illustration of a hydrocarbon contaminant plume showing the spatial distribution of anaerobic degradation processes and associated redox zones in groundwater. The plume develops as contamination migrates with groundwater flow, creating distinct zones where different terminal electron-accepting processes dominate. This figure is based on the plume fringe concept, which proposes that overlapping redox processes occur primarily at the plume fringes due to the limited transversal dispersion of dissolved electron acceptors in porous media, rather than forming distinct longitudinal redox zonation as described in the classical model (Meckenstock et al., 2015).

1.4. Model contaminants: toluene and trichloroethene

Toluene as a representative hydrocarbon. Toluene serves as a model compound for studying the anaerobic biodegradation of monoaromatic hydrocarbons. As the least recalcitrant member of the BTEX group, it degrades readily under both oxic and anoxic conditions. At 15 °C, a United States Environmental Protection Agency (U.S. EPA) study reported higher sulfate-reducing rates for toluene than for benzene (U.S. Environmental Protection Agency & HydroGeoLogic, Inc., 1999). Anaerobic degradation pathways are well characterised (Hernández-Ospina et al., 2024; Lueders, 2017) and are summarised in Figure 1.4, which outlines the key enzymatic steps from toluene activation to complete mineralisation. In this work, singly labelled [^{13}C]-α-toluene was used for GC–IRMS analysis of released ^{13}C-labelled CO_2, which provides higher specific incorporation in strict anaerobes than fully labelled toluene (Bombach et al., 2009). Many studies report $>$50% mineralisation to CO_2 with proportionally lower assimilation into phospholipid-derived fatty acids (PLFA) (Bombach et al., 2009; Evans et al., 1992; Herrmann et al., 2010; Pelz et al., 2001; Pilloni et al., 2011; Taubert et al., 2012; Winderl et al., 2010).

Temperature can strongly influence the activity and stability of enzymes such as benzyl-succinate synthase and benzoyl-CoA reductase, which catalyse the key activation and ring reduction steps in anaerobic toluene degradation. Consequently, temperature shifts may alter both degradation rates and the dominant microbial populations, with thermotolerant or mesophilic degraders prevailing under different thermal regimes. Understanding these dynamics is essential for predicting the performance of *in situ* biodegradation processes under variable subsurface temperature conditions, such as those induced by ATES.
It should be noted that thermophilic anaerobic hydrocarbon degradation has been reported in deep subsurface environments such as oil reservoirs (Head et al., 2014); however, these systems host long-adapted microbial communities and differ fundamentally from the shallow aquifers considered here, which are dominated by psychrotolerant to mesophilic microbial communities (see Section 1.5).

Trichloroethene (TCE) as a representative chlorinated solvent. TCE is a widespread and persistent groundwater contaminant, originating from its extensive industrial use and improper disposal (Bishop et al., 1993; McCarty, 2010; Rosner & Markowitz, 2013; Wanner et al., 2018). Under anoxic conditions, reductive dechlorination proceeds sequentially through dichloroethene (DCE) isomers and vinyl chloride (VC), which often accumulate as intermediates (Carter et al., 2008; Hartmans et al., 1985; Nijenhuis et al., 2007; Squillace et al., 2004; Vogel & McCarty, 1985; Weatherill et al., 2014). Complete

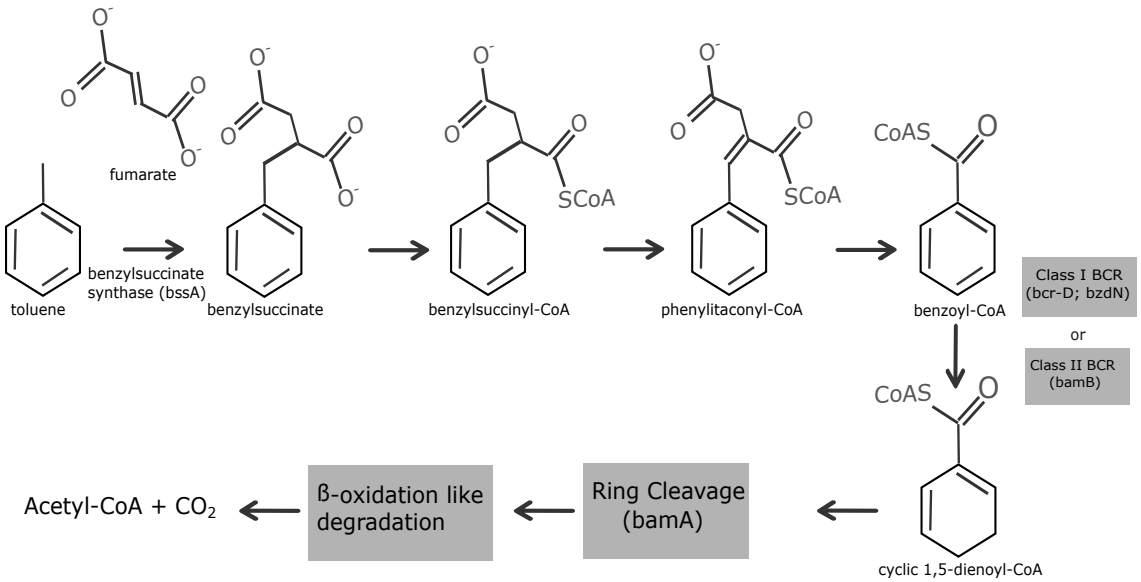


Figure 1.4.: Anaerobic degradation pathway of toluene via initial fumarate addition and subsequent benzoyl-CoA metabolism. Toluene is activated by benzylsuccinate synthase (bssA) to benzylsuccinate, followed by CoA activation to benzylsuccinyl-CoA and isomerisation to phenylitaconyl-CoA. β-oxidation–like steps yield benzoyl-CoA, which undergoes reductive dearomatisation by benzoyl-CoA reductase (BCR; Class I *bcrABCD*/*bzdN* or Class II *bamB*). Subsequent ring cleavage (bamA) and further β-oxidation produce acetyl-CoA and CO_2. Diagram adapted from Lueders (2017) and Young and Phelps (2005).

transformation of TCE to ethene typically requires specialized organohalide-respiring bacteria such as *Dehalococcoides* or *Dehalogenimonas*, but this process frequently stalls at DCE or VC when key *rdhA* genes are absent or when competition for electron donors is high (Adrian & Löffler, 2016; Dolinova et al., 2017; Fiedler & Gilbert, 2013; Holliger et al., 1998; Jugder et al., 2016). The sequential reduction steps and associated electron flow are illustrated in Figure 1.5, adapted from Sabeti Mohammadi et al. (2021).

Temperature tolerance of native dechlorinating consortia is critical for sustaining complete detoxification. Moderate thermal shifts, such as those induced by ATES operation, may enhance enzymatic activity and electron donor utilisation, whereas elevated or fluctuating temperatures could inhibit key dehalorespiring populations. Characterising these temperature sensitivities is fundamental for evaluating the resilience and long-term efficiency of *in situ* bioremediation under thermally altered subsurface conditions.

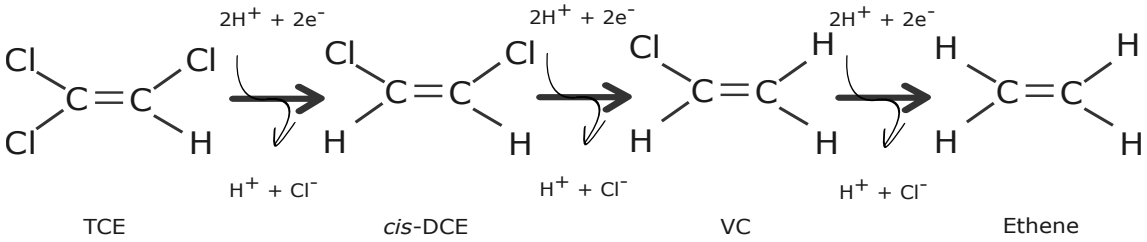


Figure 1.5.: Sequential reductive dechlorination pathway of chlorinated ethenes. Trichloroethene (TCE) can be produced by reductive dechlorination of tetrachloroethene (PCE), a fully chlorinated and common starting compound in this sequence. TCE is stepwise reduced via cis-dichloroethene (cis-DCE) and vinyl chloride (VC) to non-chlorinated ethene through reductive dehalogenation reactions, each coupled to the uptake of reducing equivalents ($2H^+ + 2e^-$) and the release of chloride ions (Cl^-). Complete detoxification requires full dechlorination to ethene, typically mediated by specialized organohalide-respiring bacteria such as *Dehalococcoides* spp. Diagram adapted from (Sabeti Mohammadi et al., 2021).

Together, toluene and TCE represent two chemically distinct but environmentally significant contaminant classes—hydrocarbons and chlorinated ethenes—whose anaerobic degradation relies on specialised microbial guilds and tightly coupled redox processes. Both pathways are highly sensitive to environmental factors, particularly temperature, which modulates enzymatic reaction rates, microbial community composition, and overall biotransformation efficiency (Beyer et al., 2016; Fletcher et al., 2011; Head et al., 2014). In the context of ATES, where subsurface temperatures can fluctuate seasonally or remain elevated for prolonged periods, understanding these temperature dependencies is crucial

for predicting the long-term behaviour and resilience of contaminant-degrading aquifer microbiomes.

1.5. Temperature dependence

Temperature fundamentally influences microbial diversity, activity, and the stability of cellular components such as enzymes, membranes, and nucleic acids (Chambon et al., 2013; Dettmer, 2002; Fiedler & Gilbert, 2013; Hantschel et al., 1995; Jesußek et al., 2013a; Jesußek et al., 2013b; Jung et al., 2011; Lee et al., 2016; Moon et al., 2023; Shapiro, 2017; Sun et al., 2022; Wei et al., 2016; Yvon-Durocher et al., 2010; Zhang & Gross, 2021). Hydrocarbon degradation occurs across a wide thermal spectrum—from 0–7 °C in sub-Antarctic soils to 80–90 °C in deep reservoirs (Delille et al., 2004; Head et al., 2014). Anaerobic hydrocarbon degradation under sulfate- or methanogenic conditions has been demonstrated at 45–75 °C for short-chain alkanes (Gieg et al., 2010; Khelifi et al., 2014; Kniemeyer et al., 2007; Mbadinga et al., 2012; Rueter et al., 1994; Zhou et al., 2012). It should be noted that sulfate reduction is physiologically possible at much higher temperatures, with active sulfate-reducing communities reported above 100 °C in deep-sea hydrothermal sediments (Jørgensen & Isaksen, 1990; Jørgensen et al., 1992). However, such systems host highly specialised thermophilic assemblages and differ fundamentally from shallow aquifers relevant to ATES.

Anaerobic degradation of BTEX compounds, particularly toluene, has been observed across a broad mesophilic range, typically between 10–40 °C, with optimal activity for many sulfate-reducing and nitrate-reducing toluene degraders reported around 20–30 °C (Hernández-Ospina et al., 2024; Lueders, 2017; U.S. Environmental Protection Agency & HydroGeoLogic, Inc., 1999). Thermophilic anaerobic BTEX degradation has also been demonstrated at temperatures of 45–75 °C in deep subsurface systems such as oil reservoirs, where long-adapted microbial consortia prevail (Chen & Taylor, 1997). However, such thermophilic systems differ fundamentally from shallow aquifers, which are typically dominated by psychrotolerant to mesophilic communities.

In addition to aromatic hydrocarbons, acetate was used here as an additional model compound to assess the temperature sensitivity of central anaerobic carbon metabolism. Acetate is mineralised under anoxic conditions across a broad temperature range. Psychrophilic methanogenic communities can utilise acetate at temperatures as low as 3–8 °C (Lettinga et al., 1999), while efficient turnover under sulfate-reducing conditions is commonly observed around 30 °C (Muthumbi et al., 2001). In thermophilic systems, mineralisation

continues at around 55 °C, predominantly via syntrophic acetate oxidation (Petersen & Ahring, 1991). Experiments with aquifer sediments further indicate acetate utilisation under elevated subsurface temperature conditions relevant to ATES (Westphal et al., 2017).

Temperature also affects the activity and stability of key enzymes involved in anaerobic aromatic hydrocarbon degradation. For toluene, enzymes such as benzylsuccinate synthase and benzoyl-CoA reductase catalyse the initial activation and subsequent ring reduction steps. Thermal shifts may therefore alter both degradation rates and the dominance of mesophilic or thermotolerant degraders, depending on whether the prevailing temperature favours the stability and function of these enzyme systems. These mechanistic constraints are important for predicting *in situ* biodegradation performance under variable subsurface temperature conditions, including those influenced by ATES.

For chlorinated ethenes, complete TCE→ethene dechlorination typically occurs at 20–30 °C for *Dehalococcoides*, whereas at 40 °C, VC accumulation is common and at ≥45 °C, activity is strongly inhibited (Fletcher et al., 2011; Löffler et al., 2013). While raising temperature generally accelerates reaction rates (e.g., 10→30 °C increases by ∼10×; 10→25 °C by 9–13×) (Friis et al., 2007; Ni et al., 2015), maintaining function requires staying within the thermal tolerance of the responsible microbial consortia to avoid incomplete biotransformation or toxic intermediate buildup.

1.6. Analytical methods (brief overview)

Mineralisation was quantified as ^{13}C-labelled CO_2 using GC–IRMS (referenced to the Vienna Pee Dee Belemite (VPDB) scale) and calculated using the Dorer equation to determine fractional mineralisation (Dorer et al., 2016). Dissolved sulfide was determined spectrophotometrically using the methylene blue method (Cline, 1969; Müller et al., 2009). Chlorinated ethenes and gaseous products were quantified by GC–FID with compound-specific calibration curves (McNair & Miller, 1998; Nijenhuis et al., 2007, 2013; Otson & Williams, 1982). Microbial community composition (16 Svedberg ribosomal ribonucleic acid (16S rRNA) and *mcrA* genes) was analysed using Illumina MiSeq sequencing, and sequence data were processed in QIIME,2 with SILVA taxonomic assignment (Caporaso et al., 2010; Hall & Beiko, 2018; Illumina, 2013; Yilmaz et al., 2014).

Detailed descriptions of all analytical and molecular procedures, including instrumentation settings, calibration, and data processing steps, are provided in the respective publication chapters and in Appendix A.

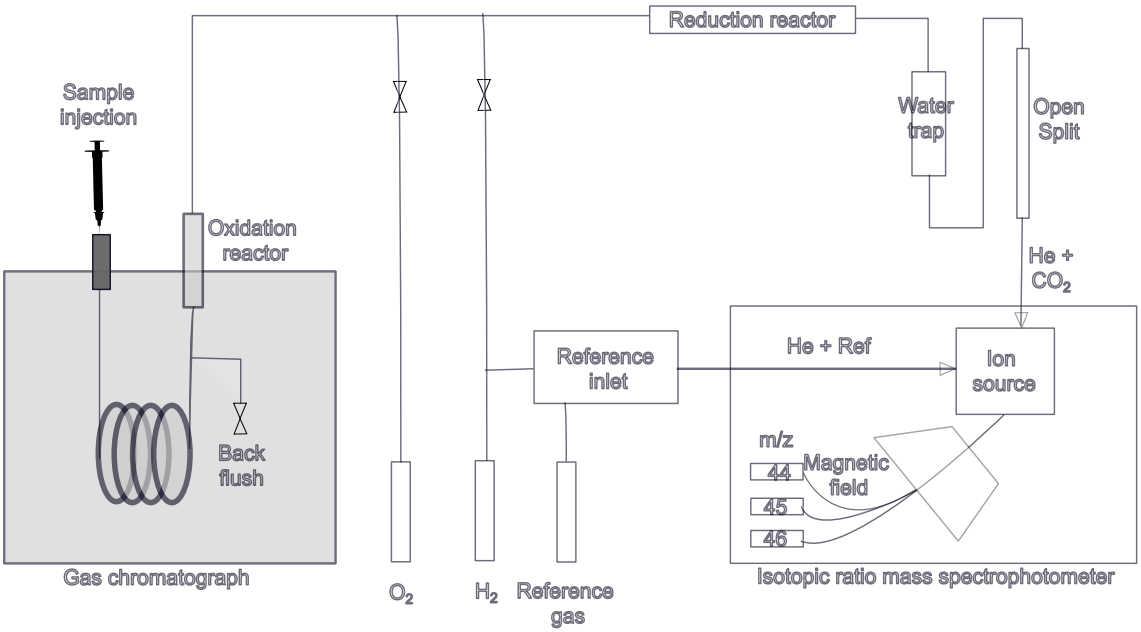


Figure 1.6.: Simplified schematic of the GC–IRMS system used for stable isotope analysis of ^{13}C-labelled CO_2. Gas samples are separated by gas chromatography, oxidised to CO_2, dried, and introduced into the isotope ratio mass spectrometer for detection of isotopes at m/z 44–46. The system includes a combustion interface and continuous-flow connection for compound-specific isotope measurement. Adapted from Pu et al. (2013).

1.7. Gaps and objectives

The sustainable design and operation of future ATES systems require a comprehensive understanding of how subsurface microbial communities respond to thermal perturbation. In particular, it is essential to identify the temperature ranges within which bioremediation and other ecosystem functions can be maintained. However, existing knowledge remains limited with respect to (i) the long-term effects of permanent warming in zones adjacent to warm wells, (ii) the impacts of recurrent seasonal temperature cycling between warm and cold wells, and (iii) the transition zone between mesophilic and thermophilic conditions, where thermotolerant lineages may confer resilience to transient heat exposure. Establishing such a site-specific, microbially informed temperature window could enable higher thermal storage capacities while preserving essential biogeochemical and contaminant-degrading functions within ATES-impacted aquifers.

Aims and objectives

This thesis investigates the influence of temperature on key anaerobic biotransformation processes in aquifer sediments, focusing on (i) toluene mineralisation, (ii) acetate mineralisation as an important metabolic intermediate, and (iii) the reductive dechlorination of TCE. The work draws on enrichment cultures from hydrocarbon-contaminated sites and sediments from a chlorinated-ethene-contaminated aquifer.

The overarching aim is to elucidate the temperature boundaries governing microbial activity and resilience under sulfate-reducing and dechlorinating conditions relevant to ATES systems. To address this aim, the following specific objectives were pursued:

Objective 1. Examine microbial community behaviour across micro-oxic and sulfidogenic phases at different temperatures using [^{13}C]-acetate mineralisation in microcosms (12, 25, 38, 45, 60, 80 °C), with simultaneous monitoring of ^{13}C-labelled CO_2 and sulfate-to-sulfide conversion, complemented by 16S rRNA gene sequencing. (Chapter 2)

Objective 2. Investigate acetate mineralisation kinetics and associated microbial taxa in a 45 °C sulfate-reducing enrichment culture (Zeitz), including both permanent and temporary temperature shifts (12, 25, 38, 60 °C) and subsequent recovery at 45 °C, by tracking ^{13}C-labelled CO_2 evolution, sulfide production, and end-point community composition. (Chapter 3)

Objective 3. Assess the effects of permanent and temporary temperature shifts on toluene mineralisation and sulfate reduction in enrichment cultures derived from from hydrocarbon-contaminated sites Zeitz (12, 25, 38 °C) and Weißandt-Gölzau (12, 25, 38,

45 °C), including constant-temperature reference incubations for rate comparison and microbial community profiling. (Chapter 4)

Objective 4. Synthesize current knowledge on the temperature dependence of chlorinated-ethene dechlorination based on laboratory and field studies, encompassing isolates, enrichment cultures, microcosms, and mesocosms, with emphasis on interactions with methanogenesis and fermentation processes. (Chapter 5)

Objective 5. Evaluate the effects of temperature (10–60 °C) on TCE reductive dechlorination and methanogenesis in TCE-contaminated Ferrara aquifer sediments amended with lactate as an electron donor, by quantifying the sequential transformation TCE → cis-DCE → VC → ethene and methane production, together with associated shifts in microbial community composition. (Chapter 6)

Together, these five objectives establish a coherent framework for understanding how temperature governs anaerobic microbial processes and contaminant biotransformation in aquifer systems. By integrating experimental, enrichment-based, and literature-derived evidence, this thesis provides both mechanistic insight and applied relevance for the sustainable implementation of ATES in contaminated and uncontaminated subsurface environments.

Chapter 2.

Effect of temperature on acetate mineralization kinetics and microbial community composition in a hydrocarbon-affected microbial community during a shift from oxic to sulfidogenic conditions

Note. This chapter presents the work published in: Bin Hudari, M. S., Vogt, C., & Richnow, H. H. (2020). *Effect of temperature on acetate mineralization kinetics and microbial community composition in a hydrocarbon-affected microbial community during a shift from oxic to sulfidogenic conditions*. *Frontiers in Microbiology*, 11, 606565. https://doi.org/10.3389/fmicb.2020.606565.



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Effect of Temperature on Acetate Mineralization Kinetics and Microbial Community Composition in a Hydrocarbon-Affected Microbial Community During a Shift From Oxic to Sulfidogenic Conditions

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Aquifer thermal energy storage (ATES) allows for the seasonal storage and extraction of heat in the subsurface thus reducing reliance on fossil fuels and supporting decarbonization of the heating and cooling sector. However, the impacts of higher temperatures toward biodiversity and ecosystem services in the subsurface environment remain unclear. Here, we conducted a laboratory microcosm study comprising a hydrocarbon-degrading microbial community from a sulfidic hydrocarbon-contaminated aquifer spiked with ^{13}C-labeled acetate and incubated at temperatures between 12 and 80°C to evaluate (i) the extent and rates of acetate mineralization and (ii) the resultant temperature-induced shifts in the microbial community structure. We observed biphasic mineralization curves at 12, 25, 38, and 45°C, arising from immediate and fast aerobic mineralization due to an initial oxygen exposure, followed by slower mineralization at sulfidogenic conditions. At 60°C and several replicates at 45°C, acetate was only aerobically mineralized. At 80°C, no mineralization was observed within 178 days. Rates of acetate mineralization coupled to sulfate reduction at 25 and 38°C were six times faster than at 12°C. Distinct microbial communities developed in oxic and strictly anoxic phases of mineralization as well as at different temperatures. Members of the Alphaproteobacteria were dominant in the oxic mineralization phase at 12–38°C, succeeded by a more diverse community in the anoxic phase composed of Deltaproteobacteria, Clostridia, Spirochaetia, Gammaproteobacteria and Anaerolinea, with varying abundances dependent on the temperature. In the oxic phases at 45 and 60°C, phylotypes affiliated to spore-forming Bacilli developed. In conclusion, temperatures up to 38°C allowed aerobic and anaerobic acetate mineralization albeit at varying rates, while mineralization occurred mainly aerobically between 45 and 60°C; thermophilic sulfate reducers being active at temperatures $>$ 45°C were not detected. Hence, temperature may affect dissolved organic carbon mineralization rates in ATES while the variability in the microbial community composition during the transition from micro-oxic to sulfidogenic conditions highlights the crucial role of electron acceptor availability when combining ATES with bioremediation.

Keywords: aquifer thermal energy storage, ATES, bioremediation, microbial community, acetate, sulfate reduction

INTRODUCTION

Aquifer thermal energy storage (ATES), leveraging on seasonal heat storage and extraction, promises a more sustainable alternative to reduce fossil fuels dependence for energy supply, especially in the heating and cooling sector (Elsland et al., 2017). For example, this sector serves residential and industrial consumers by providing solutions to energy demands for space or process heating and cooling. ATES allows for the storage of excess thermal energy during the warm summer to meet higher thermal energy demands in the cooler seasons. ATES operating temperatures vary from one installation to another (Fleuchaus et al., 2018), although they typically function under an upper temperature limit as regulatory threshold, e.g., 25°C, adopted by many Low-Temperature ATES (LT-ATES) operations (Drijver et al., 2012). Operating above this temperature threshold, e.g., up to 60°C and higher, as in the case of High Temperature ATES (HT-ATES), has since garnered interest as authorities sought to diversify their sources of energy supply. Sites where ATES systems are situated are usually urban or industry areas which are frequently loaded with contaminants released by former anthropogenic activities and thus limited as resources for drinking water. Contaminants commonly found in urban aquifers consist of petroleum hydrocarbons introduced via spillage or improper disposal (Rooney-Varga et al., 1999; Da Silva and Alvarez, 2004; Vogt et al., 2007), where their persistence remains a big hurdle to remediation up to today (NARA, 2018). In the subsurface, microbial biodegradation is crucial since contaminants can be mineralized to harmless products (e.g., carbon dioxide and water), whereas remediation techniques based on abiotic processes are usually limited in their ability to mineralize contaminants (EPA, 1999). Most hydrocarbons are biodegradable at oxic and anoxic conditions, in aquifers, however, anaerobic biodegradation is the more important attenuation process since conditions turn rapidly anoxic once oxygen is consumed. Anaerobic hydrocarbon degradation pathways are well-known and are usually carried out by microbial consortia (Mancini et al., 2002; McKelvie et al., 2005; Gieg et al., 2010; Winderl et al., 2010; Fuchs et al., 2011; Meckenstock et al., 2015; Lueders, 2017). Acetate is a key intermediate especially during syntrophic hydrocarbon biodegradation. It serves a dual role as an electron donor and a soluble carbon source which can be easily transported and utilized by microorganisms (Wu et al., 1997; Ho et al., 2002; Heuer et al., 2010; Head et al., 2014), both aerobes (Wu et al., 1997) and anaerobes (Sansone and Martens, 1982; Schink and Stams, 2006; Heuer et al., 2010). At anoxic conditions, sulfate is a main electron acceptor in marine but also freshwater habitats making dissimilatory sulfate reduction a commonplace in contaminant degradation (National Research Council, 2000; Miao et al., 2012).

Generally, biodegradation processes accelerate at higher temperature leading to the hypothesis that organic contaminants may be preferentially degraded at elevated temperatures during ATES operation, enabling the possibility to combine *in situ* bioremediation and ATES. However, unlike Arrhenius-like models where biochemical rates tend to increase with temperature (Mohr and Krawiec, 1980; Huang et al., 2011), the temperature-microbial growth paradigm is somewhat different, i.e., species are categorized according to their maxima, minima, and optimal growth temperatures (Dettmer, 2002). In general, growth ranges of psychrophiles, mesophiles, thermophiles and hyperthermophiles are: 0–20, 8–48, 40–70, and 65–90°C, respectively (Madigan et al., 2017). Optimal temperatures allow maximal growth rates; rates will be significantly lower and finally cease when temperatures reach sub-optimal and boundary conditions. This is paramount since ATES are likely to drive seasonal temperature fluctuations in the subsurface. As such, temperature fluctuations from ATES could influence the structure of the indigenous microbial community, its biodegradation potential, as well as the physicochemical characteristics of the contaminants such as adsorption and solubility (Koproch et al., 2019).

In ATES, it is postulated that while temperatures < 40°C may enhance biodegradation by increasing microbial activities of mesophiles, temperatures as high as 80°C or above may considerably affect the structure and function of subsurface microbial communities and adversely impact biodegradation. Nonetheless, the mineralization capacity of the indigenous microbial community is imperative in a comprehensive assessment of combined ATES and remediation potential. While the effects of temperature on the degradation of hydrocarbons and its key intermediate acetate have been widely reported, temperatures are not specifically geared toward ATES-related and microbial growth temperature targets. Moreover, not much has been reported on microbial community shifts during the transition from an oxic to an anoxic environment. Henceforth, in this study, the effects of temperature on (i) extent and rates of acetate mineralization by a hydrocarbon-adapted microbial community at ATES-relevant temperatures and (ii) temperature-induced shifts in the microbial community structure are addressed.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of pro analysis quality and purchased from Sigma-Aldrich (United States), Merck (Germany) and Carl Roth (Germany) unless stated otherwise. Uniformly ^{13}C-labeled sodium acetate was purchased from Cambridge Isotope Laboratories Inc. (98% purity, 99 atom%, Massachusetts, United States). *N,N*-dimethyl-1,4-diphenylenediammonium dichloride (DMPD) was obtained from Merck (Switzerland).

Experimental Setup

For our experiment, we used coarse sand taken from columns of an *on-site* system percolated with sulfidic benzene-contaminated groundwater for 10 years and 6 months at a mean temperature of 15°C as described elsewhere (Vogt et al., 2007; Taubert et al., 2012). The coarse sand was collected in January 2017 and maintained since then in 1 L Schott bottles filled with anoxic bicarbonate-buffered mineral salt media comprising 10 mM sulfate as electron acceptor and supplemented with

10 μL of benzene dissolved in 15 mL of the inert 2,2,4,4,6,8,8-heptamethylnonane (HMN) as a carrier phase. These bottles were incubated at room temperature (~20°C) and were re-supplemented with benzene and sulfate if substrates had been consumed. For this study, microcosms were prepared in October 2018 using the coarse sand material from one of the bottles in an anaerobic glove box (95% N_2/5% H_2 in normal operation, Coy Laboratory Products, United States). Each microcosm comprised of 40 g of gravel and 80 mL of modified mineral salt medium (Bak and Pfennig, 1991) in 120 mL serum bottles which were gas-tight sealed with Teflon-coated butyl rubber stoppers (Wheaton Industries, United States) and crimped aluminum caps. The recipe of the modified mineral salt medium is described in the **Supplementary Table S1**.

A total of 65 replicate bottles were set up in the glove box distributed amongst six temperature settings: 12°C (11 bottles), 25°C (12 bottles), 38°C (12 bottles), 45°C (12 bottles), 60°C (11 bottles), and 80°C (7 bottles). Oxygen was accidentally introduced during this step arising from a technical incident and a faulty oxygen sensor. For each incubation temperature, three bottles were used as replicates for analytical measurements during the whole incubation period and two for sterile controls for the same purpose. The remaining replicates were sacrificed at various time points of the incubation period for microbial community analysis. Replicates incubated at 12, 25, 38, and 45°C were finally sacrificed when acetate mineralization was completed based on sulfide and $\delta^{13}CO_2$ data. Prior to sodium acetate amendments, all microcosms were incubated at 12, 25, 38, 45, 60, and 80°C for 3 days to guarantee that microcosm had reached the experimental temperatures before acetate addition. All replicates were amended with uniformly ^{13}C-labeled sodium acetate by a plastic syringe (B Braun, Germany) to a final concentration of 1 mM from a pre-prepared sterile anoxic stock solution (100 mM), except for two replicates at 12°C (sacrificed on days 214 and 220) which were further maintained by the addition of 750 μL of the stock solution of ^{13}C-labeled acetate (100 mM). Microcosms were incubated horizontally and statically in the dark at 12, 25, 38, 45, 60, or 80°C using various incubators (IN55, Memmert, Germany; Heraeus incubator, Thermoscience, United States). Samples and headspace collection vials (10 mL) for chemical analyses over the course of the experiment were conducted by means of sterile, nitrogen-flushed syringes. Headspace collection vials were sealed with Teflon-coated butyl rubber stoppers and aluminum crimps. All used glassware was sterilized by autoclaving (121°C, 20 min) before usage.

Chemical Analyses

Analysis of $\delta^{13}CO_2$

Headspace samples (4 mL) were collected over the course of the experiment using N_2-flushed syringes into N_2-flushed (2 min, 1 bar) collection vials (10 mL) which were sealed with Teflon-coated butyl rubber stoppers and aluminum crimps. The carbon isotope ratios of headspace CO_2 and CH_4 were measured by gas chromatography-combustion-isotopic ratio mass spectrometry (GC-IRMS) comprising a gas chromatograph (Agilent, United States) coupled to a mass spectrometer via a Conflow III Interface (Germany). The column used for the measurement was a PoraplotQ column (25 m × 0.32 mm ID, 1 μm film; Chrompack, Middleburg, United States). Carbon dioxide separation was achieved isothermally at 40°C using helium as the carrier gas at a flow rate of 2 mL min^{-1}, with an expected elution time of 500 s after injection. After the separation, the sample was transferred to a combustion furnace (GC/C-III; Thermo Fisher Scientific, Bremen, Germany) held at 980°C on a CuO/Ni/Pt catalyst to track also carbon isotope signatures of methane which would have been converted to carbon dioxide. However, methane was never detected in any of the measured samples. Samples of different volumes (100–500 μL) were injected at 1:5 split ratios. Carbon isotope signatures are given in delta notation (per mill, ‰) relative to the Pee Dee Belemnite standard.

The percent of mineralization (MIN%) of each replicate was calculated according to the following equation (Dorer et al., 2016):

$$MIN\ (\%) = \frac{\left[HCO_3^-\right] \times R_{VPDB} \times (\delta_{end} - \delta_{start})}{(n) \times [acetate] \times (1 + R_{VPDB} \times (1 + \delta_{(start)}))} \times 100 \quad (1)$$

This takes into account the starting (δ_{start}) and endpoint (δ_{end}) isotope values (‰), bicarbonate (HCO_3^-) and acetate concentration and number of labeled carbon of the target compound, where in the case of fully labeled acetate, n = 2. This allows a relative comparison of the extent of acetate mineralization.

Analysis of Sulfide

Sulfide was quantified in liquid samples using the modified methylene blue method originally described elsewhere (Cline, 1969; Muller et al., 2009). Samples (200 μL) were collected over the course of the experiments via sterile N_2-flushed plastic syringes and immediately added to 1 mL of 3% zinc acetate dihydrate (Merck, Germany) in glass test tubes. Four mL of bi-distilled water was added followed by the addition of 400 μL of Cline reagent, which was prepared in an amber Schott bottle by dissolving (per 50 mL) 1 g of *N,N*-dimethyl-1,4,-diphenylenediammonium dichloride (Merck, Switzerland) and 1.5 g $FeCl_3 \times H_2O$ (Honeywell, United States) in 25% HCl (Merck, Germany). The mixture was incubated in the dark for 20 min, subsequently vortexed and transferred to 2.5 mL cuvettes in which the absorbance at 670 nm was determined with a spectrophotometer (Novaspec III, Amersham Biosciences, Cambridge, United Kingdom) using samples prepared with bi-distilled water as a blank.

Microbial Community Analysis

Microcosms were sacrificed randomly over a time course from each treatment temperature. Sessile cells were harvested from the coarse sand material by a series of sodium tetrapyrophosphate treatment using a modified method described by Velji and Albright (1984). Firstly, 0.6 mL of cold, sterile 1% sodium tetrapyrophosphate (Sigma–Aldrich, United States) was added to each microcosm bottle to give an approximate final concentration

of 0.01% sodium tetrapyrophosphate; the mixture was sonicated for 1 min in a sonicating bath (Bandelin, Germany). The liquid was transferred into a new 50 mL Falcon tube. The step was repeated at least three times: two rounds of sonication with 5 mL of 1% sodium tetrapyrophosphate for 1 min followed by a final round of 5 mL of 1% sonication for five min. After each round of sonication, the liquid was transferred into a Falcon tube and kept cool in an ice bath. At the end of the sonication step, the Falcon tubes were centrifuged at 11.000 rpm at 4°C (Type 5804R, Eppendorf, Germany). The supernatant was discarded and the pellet was stored at −20°C in the freezer until DNA extraction. DNA was extracted from thawed cell-sand debris pellets with the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was eluted in 40 μL elution buffer. Extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Malaysia) with the Qubit HS (High Sensitivity) Assay kit (Thermo Fisher Scientific, United States).

16S rRNA genes were amplified using the Klindsworth primer pair (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) generating an amplicon of 464 bp (Klindworth et al., 2013). The PCR was carried out according to the following protocol (preparation and program) in an S1000 Thermal Cycler (Bio-Rad, United States). Each PCR reaction mixture (25 μL volume) consisted of 12.5 μL of MyTaq (Bioline), 1 μL of each primer 341F/785R (10 pM), 1 μL of Bovine Serum Albumin (1:20) and up to 15 ng of DNA, quantified via the Qubit 3.0 Fluorometer. The PCR program based on 10–15 ng of DNA comprised of an initial activation step of 95°C for 3 min, 25 cycles of: 30 s at 95°C for denaturation, 30 s at 55°C for annealing, 30 s at 72°C, and a final extension step at 72°C for 5 min. For DNA samples containing less than 10 ng, 30 or 35 cycles were used instead of 25. The 16S PCR products were verified via gel electrophoresis (1.5% agarose) and purified with Ampure XP beads (ThermoFisher, United States), and verified again via gel electrophoresis. Sequencing libraries were prepared using the Illumina MiSeq Reagent Kit v3 (2 × 300 bp) as recommended by the manufacturer on 16S Metagenomic Sequencing Library Preparations (Illumina, 2013). Briefly, multiplex indexing barcodes were added using Illumina Nextera Xt Index Kit, subsequently purified by Ampure XP beads; the libraries were subsequently quantified with the Qubit BR (Broad Range) Assay kit (Thermo Fisher Scientific, United States). Purified PCR products were each diluted to 4 nM and pooled together. The pooled libraries were sequenced on an Illumina Miseq platform at the Department of Environmental Microbiology of the Helmholtz Centre for Environmental Research –UFZ. The sequences were analyzed using the QIIME 2 v2019.1 (Bolyen et al., 2019) pipeline provided by Dr. Denny Popp (UFZ, Department of Environmental Microbiology, Systems Biology of Microbial Communities Working Group). Briefly, primer sequences and adapters appended during the library preparation were removed from the de-multiplexed sequences using Cutadapt v1.18 (Martin, 2011). Sequences were further trimmed, denoised by removing low quality reads and chimeras and merged using DADA2 (Callahan et al., 2016). Amplicon sequence variants (ASVs) were assigned to bacterial DNA against the silva132 database (Quast et al., 2013; Yilmaz et al., 2014). Finally, rarefaction curves and diversity indices (Bray–Curtis Dissimilarity) were generated using the QIIME 2 software to assess if the sampling depth was sufficient and to test if there are significant differences between the incubation conditions toward the microbial community composition. For analysis of Bray–Curtis dissimilarity, samples were rarefied to the minimum sequencing read with minor losses to the total amplicon sequencing variants ASVs (**Supplementary Figure S1**). Sequences were deposited at the European Nucleotide Archive (ENA) under the primary accession number PRJEB40338.

RESULTS

Effect of Temperature on Acetate Mineralization

Acetate was mineralized at all temperatures, at varying degrees and different rates. In **Figures 1A,B**, data for $\delta^{13}CO_2$ and sulfide concentrations for the six different temperatures are shown. These values were derived from triplicate cultures, each incubation temperature was set up with at least eight replicates which were sacrificed for biodiversity analysis at different time points. The $\delta^{13}CO_2$ composition and sulfide concentration were monitored for all replicates until they were sacrificed. These data were used to derive the percentage of mineralization and concentrations of acetate utilized and sulfide produced (**Table 1**). Generally, $\delta^{13}CO_2$ and sulfide values of all replicates behaved similarly to the data of the triplicates shown in **Figure 1** within each temperature setting unless stated otherwise. Phase 1 is characterized by the evolution of $^{13}CO_2$ until a plateau was reached within 7–28 days (**Figure 1C** and **Table 1**). Increases of $\delta^{13}CO_2$ values due to $^{13}CO_2$ formation by $^{13}C_2$-acetate oxidation were observed at all temperatures within 24–48 h (data not shown), illustrating an immediate begin of acetate turnover. At 80°C, only one replicate mineralized acetate, albeit in a limited way, as observed during the first 7 days of incubation (**Table 1**). Above all, this phase was considered as a putative aerobic phase since no sulfide was detected in this period.

Rates of acetate mineralization were calculated to compare between the short burst of activity in the putatively aerobic phase 1 and the prolonged anaerobic phase 2 which are represented in **Table 1**. Phase 1 mineralization rates were found to be similar at 25°C (75.0 ± 34.3 μM d^{-1}), 38°C (74.1 ± 20.2 μM d^{-1}), and 60°C (71.4 ± 24.3 μM d^{-1}), with no apparent lag phase (**Table 1**). The degree of mineralization was also similar at 60°C (16.4–19.8% mineralized), 25°C (13.8–22.4%), and 38°C (15.3–17.6%). At 45 and 12°C, the rates of acetate mineralized were lower than that observed at 25, 38, and 60°C, with average values of 10.7 ± 8.2 μM d^{-1} and 12.2 ± 6.9 μM d^{-1}, respectively. At 12°C, the degree of mineralization was significantly lower than observed at 25–60°C (**Table 1**). Mineralization at 80°C was observed in only one of three replicates and to a small degree (53.8 μM or 5.38% acetate mineralized, **Table 1**) with a rate of 7.7 μM d^{-1}.

The second acetate mineralization phase (phase 2) was characterized by sulfide production; this started after a certain period of no $\delta^{13}CO_2$ increment (plateau phase) which indicated

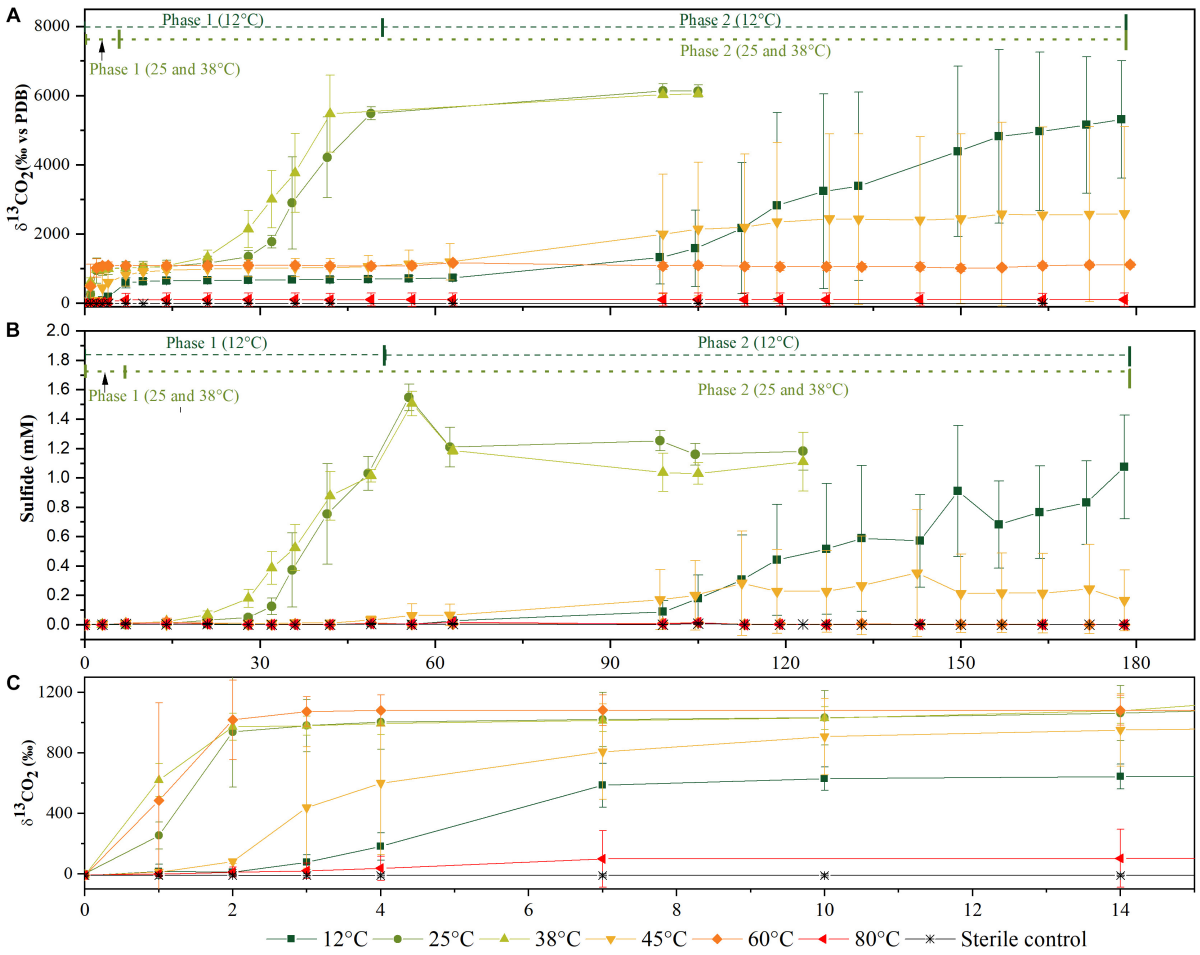


FIGURE 1 | Time courses of ^{13}C-labeled CO_2 production **(A)** and of sulfide production **(B)** from microcosms incubated at different temperatures in the aerobic phase 1 and anaerobic phase 2 of mineralization. Zoom in of the time course during the first 15 days of **(A,B)** for ^{13}C-labeled CO_2 production **(C)** for higher resolution of mineralization phase 1. Data represented as an average of three biological replicates. Error bars represent ± standard deviations of the triplicate values.

the end of the first mineralization phase (**Figures 1A,B**). For triplicate microcosms incubated at 12, 25, and 38°C, the second mineralization phase began after around 10–14 days (25 and 38°C) and 63 days (12°C), respectively (**Figure 1**). The acetate mineralization rate in phase 2 was lower at 12°C (9.8 ± 4.3 μM d^{-1}) when compared to 25 and 38°C (18.8 ± 1.5 μM d^{-1} and 22.9 ± 5.3 μM d^{-1} respectively) (**Table 1** and **Figure 2A**). Acetate mineralization was completed in less than 60 days at 25 and 38°C, surpassing $\delta^{13}CO_2$ values of 6000‰; whereas at 12°C, mineralization took longer and was completed within 214 days for all replicates (note that the curve in **Figure 1A** only represents the averaged value of the replicates and therefore only shows mineralization data up to 178 days). Sulfide production rates mirrored trends to that of acetate mineralization with the slowest rate at 12°C (8.8 ± 4.1 μM d^{-1}), whereas higher rates were observed at 25 and 38°C (21.3 ± 3.1 μM d^{-1} and 18.0 ± 4.7 μM d^{-1} respectively) (**Figure 2B**). At 45°C, the biphasic behavior was found in only one replicate, 45°C-3, with concomitant sulfide production at a rate of 5.4 μM d^{-1} (**Figure 3**). No sulfide was produced in two of three replicates at 45°C as well as in all replicates at 60 and 80°C, corresponding with absent acetate mineralization; hence, phase 2 of acetate mineralization was absent in these replicates. Sterile controls did not produce any ^{13}C-enriched CO_2 or sulfide. The molar ratio of average sulfide produced to acetate mineralized in phase 2 were 0.89 (12°C), 1.01 (25°C), 0.86 (38°C), and 0.71 (45°C-3); thus slightly less sulfide was produced relative to the observed acetate mineralized in three of four temperatures (**Table 2**). The molar ratios were tested for normality using the Shapiro–Wilk test and found to be normally distributed ($P > 0.05$). The one-way ANOVA suggests that there were no significant differences between the molar ratios, $F(1,8) = 1.6$, $P > 0.05$.

Community Compositions at Different Temperatures

The community compositions at various stages of incubation for the respective temperatures, represented at the class and genus level, are shown in **Figures 4**, **5**, respectively. The communities

Temp'	Replicate	Phase	Period (*d*)	Duration (*d*)	Acetate mineralization				Sulfide production			Theoretical oxygen consumed*	
					Mineralization (%)	Acetate mineralized (μM)	Rate (μM/d)	Average (μM/d)	Sulfide produced (μM)	Rate (μM/d)	Average (μM/d)	Concentration (μM)	Average (μM)
12°C	1		0–28	28	12.0	119.8	4.3			–		239.6	
	2	1	0–7	7	11.8	118.2	16.9	12.2 ± 6.9	Not observed	–	–	236.3	231.2 ± 11.8
	3		0–7	7	10.9	108.8	15.5			–		217.6	
	1		63–127	64	93.1	931.0	14.5		860.0	13.4		–	
	2	2	63–164	101	86.7	867.2	8.6	9.8 ± 4.3	725.5	7.2	8.75 ± 4.1	–	–
	3		63–214	151	94.1	941.0	6.2		848.7	5.6		–	
25°C	1		0–2	2	13.8	138.2	69.1			–		276.3	
	2	1	0–2	2	22.4	223.8	111.9	75.0 ± 34.4	Not observed	–	–	447.7	358.5 ± 85.9
	3		0–2	4	17.6	175.8	43.9			–		351.6	
	1		10–49	39	80.2	801.5	20.6		850.9	24.3¶		–	
	2	2	10–49	39	70.3	703.2	18.0	18.8 ± 1.5	637.0	18.2¶	21.26 ± 3.1	–	–
	3		10–49	39	69.7	696.6	17.9		744.8	21.3¶		–	
38°C	1		0–2	2	17.6	176.1	88.1			–		352.2	
	2	1	0–3	3	15.3	152.8	50.9	74.1 ± 20.2	Not observed	–	–	305.5	330.5 ± 23.5
	3		0–2	2	16.7	166.9	83.4			–		333.7	
	1		10–42	32	81.2	811.7	25.4		686.4	19.6§		–	
	2	2	10–42	32	53.6	536.1	16.8	22.9 ± 5.3	447.3	12.8§	18.04 ± 4.7	–	–
	3		10–42	32	84.8	848.0	26.5		760.2	21.7§		–	
45°C	1		0–21	21	13.7	137.1	6.5			–		274.3	
	2	1	0–28	28	15.2	151.7	5.4	10.7 ± 8.2† (*n* = 3)	Not observed	–	–	303.4	326.7 ± 67.2
	3		0–10	10	20.1	201.3	20.1	6.0 ± 0.8‡ (*n* = 2)		–		402.5	
	1		–	–	–		–		–	–		–	
	2	2	–	–	–		–	7.6	–	–	5.4	–	–
	3		42–127	85	64.7	647.1	7.6	(*n* = 1)	461.8	5.4	(*n* = 1)	–	
60°C	1		0–3	3	18.0	180.5	60.2			–		361.0	
	2	1	0–3	3	16.4	164.0	54.7	71.4 ± 24.3	Not observed	–	–	328.0	362.0 ± 34.5
	3		0–2	2	19.9	198.5	99.3			–		397.1	
	1												
	2	2	Not observed	–	–	–	–	–	Not observed	–	–	–	–
	3												
80°C	1		0–7	7	5.4	53.8	7.7			–		107.5	
	2	1	–	–	–	–	–	7.7	Not observed	–	–	–	107.5
	3		–	–	–	–	–	(*n* = 1)		–		–	(*n* = 1)
	1												
	2	2	Not observed	–	–	–		–	Not observed	–	–	–	–
	3												

**The theoretical oxygen consumption was calculated according to the equation: $CH_3COOH + 2\ O_2 \rightarrow 2CO_2 + 2\ H_2O$. ¶, § No sulfide data available for day 10. Sulfide production data and rates calculated between periods 14–49 and 7–42 for 25 and 38°C respectively. †, ‡ Calculation with/without inclusion of replicate 45°C-3 value.*

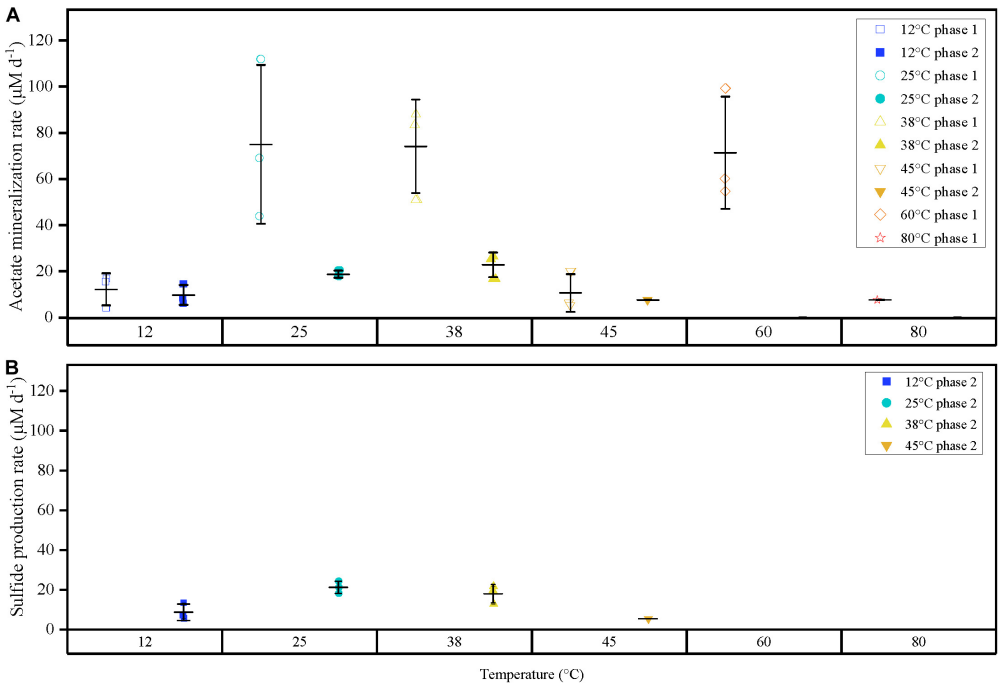


FIGURE 2 | (A) Comparison of acetate mineralization rates for triplicates for both phases 1 and 2 represented as points differentiated by temperature (color), and phase (shape). **(B)** Sulfide production rates for phase 2 observed at 12, 25, 38 and 45°C-3. Outer horizontal bars indicate the standard deviation with respect to the triplicate average (where applicable). Acetate mineralization phase 2 was not observed at 60 and 80°C. Acetate mineralization at 45°C phase 2 and 80°C phase 1 is represented by a single point, respectively. Phase 1 is characterized by a short burst of activity compared to the prolonged activity in anaerobic phase 2.

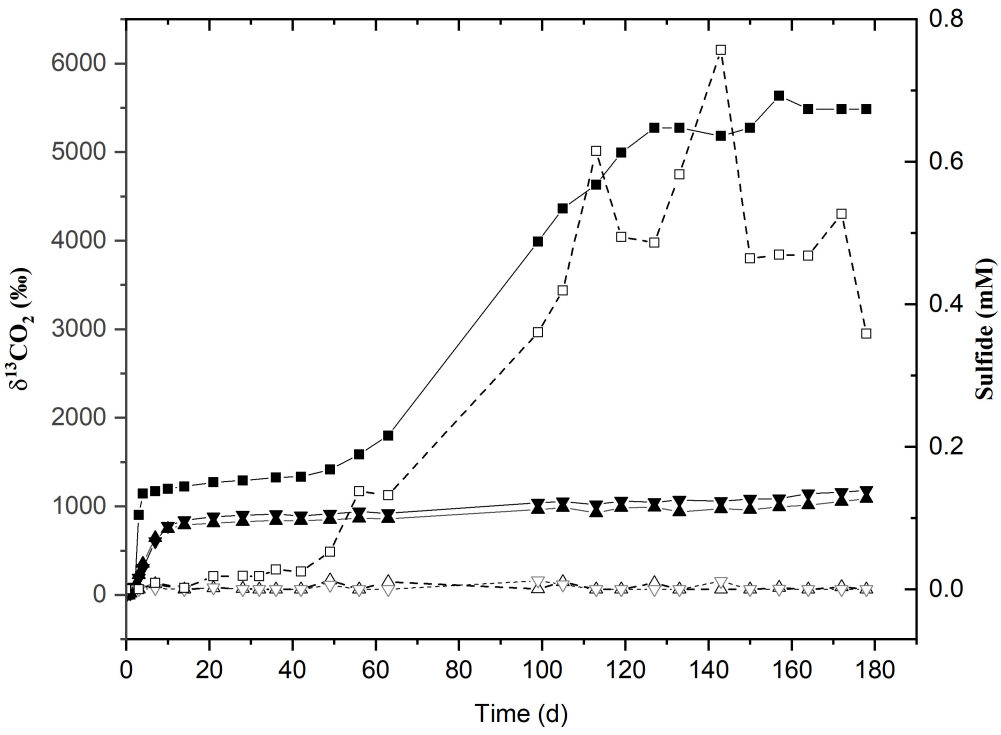


FIGURE 3 | Time course for ^{13}C-labeled CO_2 production (solid lines with filled symbols) and sulfide (dotted lines with hollow symbols) incubated at 45°C. $\delta^{13}CO_2$ and sulfide values for the sole active replicate (45°C-3) are represented by (■) and (□) respectively. (▲) and (▼) represent the $\delta^{13}CO_2$ values for replicates 45°C-1 and 45°C-2. The remaining replicates for sulfide are given as (Δ) and (∇).

TABLE 2 | Acetate mineralization, sulfide production, and the ratio of sulfide produced to acetate mineralized at 12, 25, 38, and 45°C.

Temperature	12°C	25°C	38°C	45°C
Number of replicates	3	3	3	1
Acetate mineralized	913.1 ± 40	733.8 ± 58.8	731.9 ± 170.5	647.1
Sulfide produced	811.4 ± 74.6	744.2 ± 107	631.3 ± 163.6	461.8
$\frac{\text{Sulfide produced}}{\text{Acetate mineralized}}$	0.89	1.01	0.86	0.71

from the microcosms incubated at 80°C could not be sequenced due to insufficient amounts of DNA extracted from the biomass.

The MiSeq sequencing data returned 2,446,862 16S rRNA gene sequence reads with an average number of reads of 67,968 (±21,400) per sample and minimum and maximum sequence reads per sample of 21,993 and 118,715, respectively. The rarefaction curve showed variations in the number of observed amplicon sequence variants (ASVs), varying between 50 and 450 (**Supplementary Figure S1**). The total number of sequences per sample were used to represent microbial community composition (**Figures 4**, **5**). However, for Bray-Curtis analysis (**Figure 6**), sequences of each respective sample were rarefied to the minimum of 21,993 sequences reads. The DNA extracted from the coarse sand used to set up the microcosms showed a relatively higher ASV richness, decreasing with the start of the experiment (**Supplementary Figure S1**).

In the inoculum, dominant phylotypes belonging to Anaerolineae (Chloroflexi) and Deltaproteobacteria (Proteobacteria) comprised approximately 24 and 17% of the assigned reads, respectively. Members of the Ignavibacteria (Bacteroidetes) and Clostridia (Firmicutes) were also amongst the main ASVs present, comprising 8 and 9%, respectively. On the genus level, abundant ASVs corresponded to the Anaerolinea-like *Pelolinea* (∼11.3%), as well as several other members of the Anaerolineae (∼12%), *Desulfobacca* (∼6%), *Desulfatiglans* (∼6%) within the Deltaproteobacteria, and an unspecified member of the Ignavibacteria related to the family BSV26 (7%).

Community Composition at 12, 25, and 38°C

Upon acetate mineralization in the first phase (**Figure 1**), microcosms at 12, 25, and 38°C sacrificed on days, 4, 7, and 14

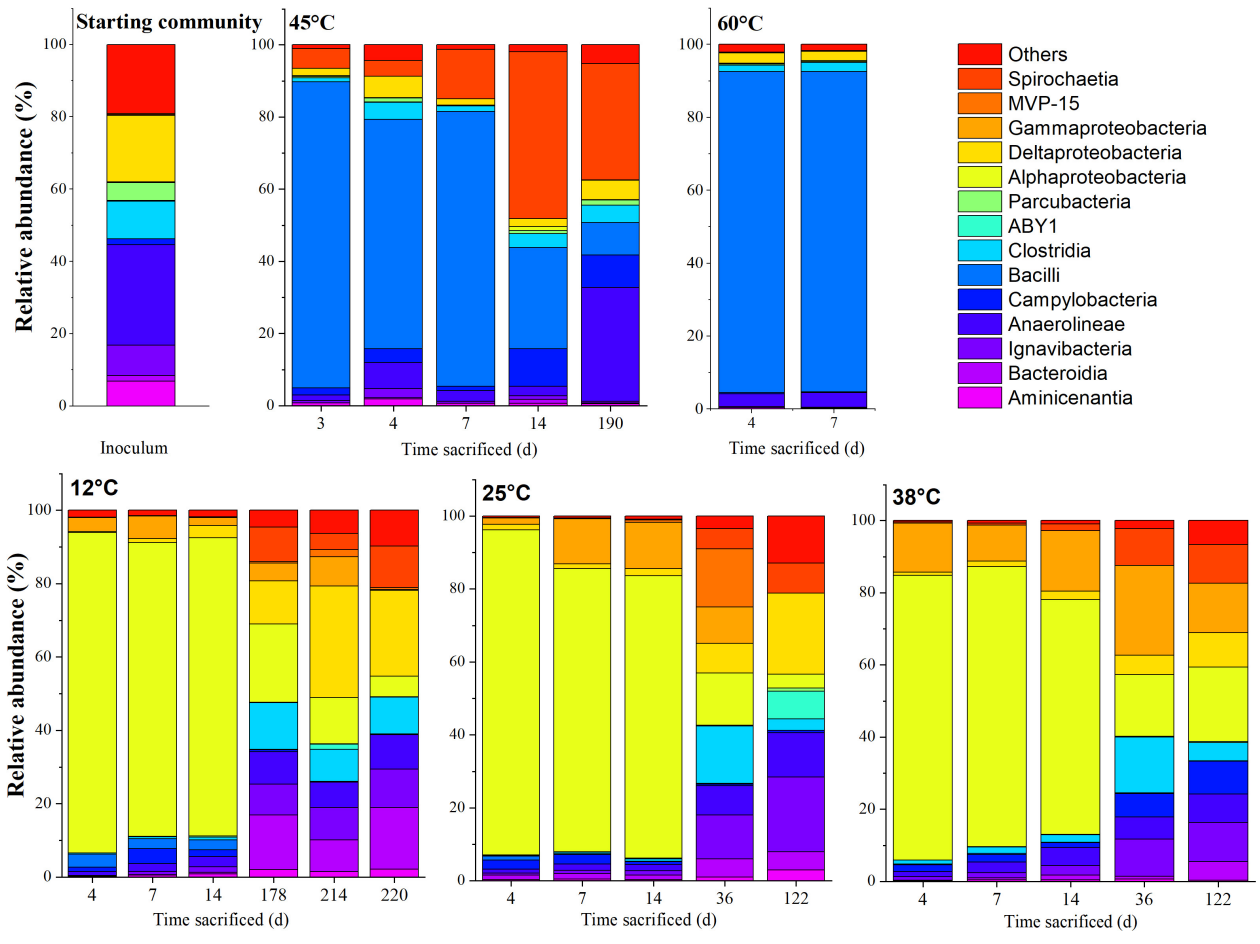


FIGURE 4 | Time-resolved distribution of the bacterial community composition of the community assigned to order. Members which constitute < 5.0% across all samples were grouped as others.

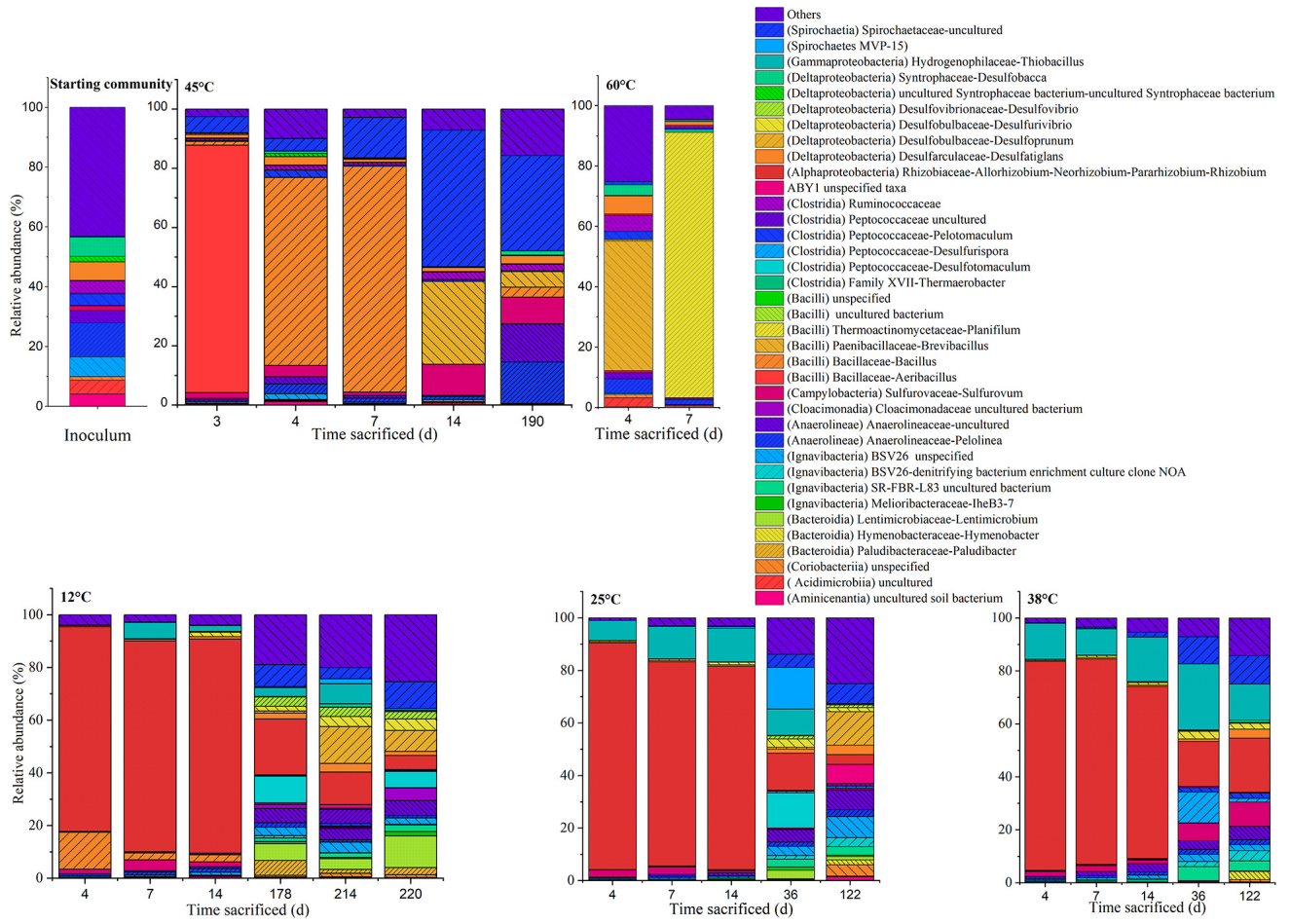
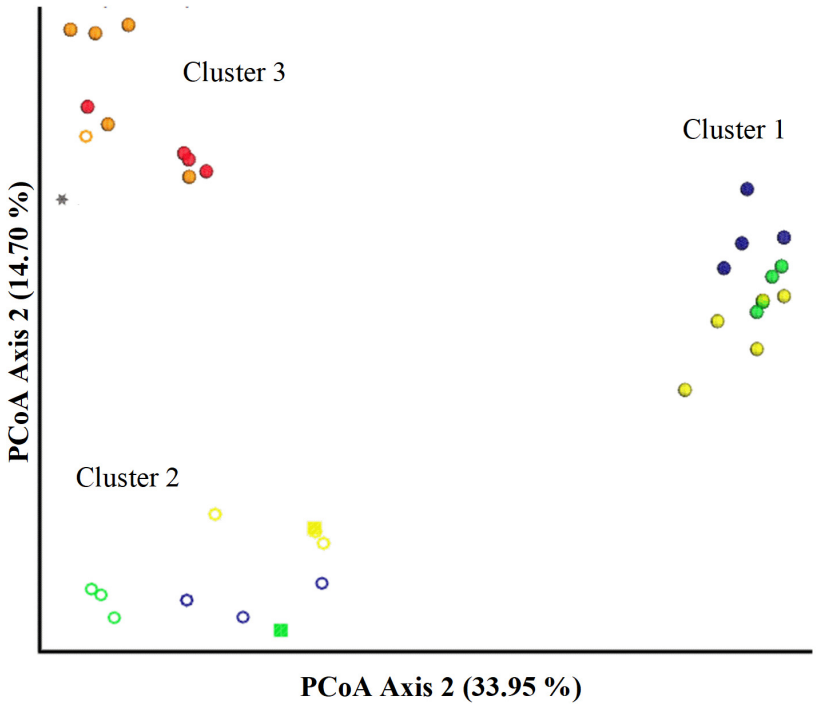


FIGURE 5 | Time-resolved distribution of the bacterial community composition assigned to genera. Members which constitute < 3.0% across all samples were grouped as others (<3.0%). The class assignments of the respective genus assigned within the parentheses.

were dominated by sequences affiliated to the mainly aerobic *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group belonging to the Alphaproteobacteria (∼65–86%) which were hardly detectable in the inoculum (0.16%). Other more abundant taxa in the first mineralization phase at 12°C to 38°C were phylotypes belonging to *Thiobacillus* (Gammaproteobacteria) (up to 16.8% compared to 0.06% in the inoculum) and *Sulfurovum* (Campylobacteria) (up to 4.1% compared to 1.6% in the inoculum). Conversely, phylotypes affiliated to *Bacillus* were more evident at 12°C (2.6–14.0%) than at 25°C (<0.3%) and 38°C (<0.3%) during the first mineralization phase.

In the second mineralization phase characterized by sulfide production (**Figure 1**), replicates at 25 and 38°C were sacrificed within 122 incubation days except for one replicate at 45°C, and triplicates at 12°C. The 12°C replicates were sacrificed on days 178, 214, and 220; the latter two replicates were sacrificed beyond the experimental duration and received an additional uniformly labeled acetate (∼1 mM) on day 178 to in order to maintain the culture until sacrifice. In spite of the different treatment, the three replicates were comparatively similar in abundance at both class and genus level. At the class level, the 12°C triplicates revealed several similarities to setups at 25 and 38°C sacrificed on days 36 and 122. Mainly, the abundant Alphaproteobacteria observed in phase 1 decreased from ∼65–86% to ∼3–21% at 12, 25, and 38°C, succeeded by a mixed community which varied amongst the three temperatures, generally represented by ASVs affiliated to the classes Ignavibacteria (Bacteroidetes), Anaerolineae (Chloroflexi), Deltaproteobacteria, Spirochaetia and Gammaproteobacteria. At 12°C, Deltaproteobacteria (10–28%) and Bacteroidia (7–16%) were the most abundant phylotypes (on days 178, 214, and 220), while at 25°C and 38°C, Ignavibacteria and Gammaproteobacteria were more abundant (on days 36 and 122). At 25°C, abundance of Ignavibacteria (10–18%), Gamma- and Deltaproteobacteria (8–21%) were comparable to that at 38°C with abundances of 10% and 5–8%, respectively.

At the genus level, in the 12°C replicates several sulfate-reducing genera belonging to the Deltaproteobacteria were more abundant: *Desulfoprunum* (0.8–14.0%), *Desulfurivibrio* (1.8–4.4%), *Desulfovibrio* (2.8–3.5%), and *Desulfatiglans* (1.5–3.2%). Other genera found to be enriched are the Bacteroidia-like *Lentimicrobium* (∼4.1–11.9%) and an uncultured member



Label	Temperature (°C)	Time of sacrifice (d)	Mineralization (%)
✱	Starting community	0	0
●	12	4-14	1-12
○	12	>36	100
●	25	4-14	13-18
■	25	36	94
○	25	122	99-100
●	38	3-14	14-18
■	38	36	59
○	38	122	90-100
●	45	3-14	1-21
○	45	190	92
●	60	4-7	9-21

FIGURE 6 | Principal coordinate plots (Bray–Curtis) of microbial communities in microcosms from different treatments and time points of sacrifice.

of the Spirochaetaceae (∼4.3–10.1%). Additionally, phylotypes affiliated to *Desulfotomaculum* (up to ∼10.0%), *Paludibacter* (1.5–5.6%) and an uncultured Anaerolineaceae member (4.3–5.6%) became apparent when conditions became sulfidic at the onset of the second phase.

At 25°C, in the second phase (day 122), sequences affiliated to the genera *Desulfoprunum* (12–14%), and an uncultured Spirochaetaceae (∼8%) were amongst the most abundant. Notably, members of *Thiobacillus* and *Sulfurovum* decreased from ∼7.5–12.7% (phase 1) to 0–7.6% (phase 2) and from ∼2.4% (phase 1) to 0.4–0.6% (phase 2), respectively.

At 38°C, the genera *Sulfurovum* (9.1%), *Desulfatiglans* (3.4%), an uncultured bacterium related to the SR-FBR-L83 family (∼5%), and Anaerolineaceae (5–12%) were some of the most abundant phylotypes. *Thiobacillus* sequences were the most abundant by day 36 (25%) but gradually decreased with time to ∼14% on day 122. Besides that, members of the Ignavibacteria (e.g., uncultured member of BSV26), Anaerolinea (e.g., *Pelolinea*), Deltaproteobacteria (e.g., *Desulfovibrio*), and Spirochaetia also experienced an uptick in abundance.

Besides the mentioned similarities in community compositions of the second phase at temperatures of 12, 25, and 38°C, some differences were observed. For example, abundances of *Desulfoprunum* members were higher at 12 and 25°C (up to 14.8%), than at 38°C (<3%), albeit after longer incubation. Members of the Ignavibacteria (e.g., belonging to the family BSV26) were most abundant at 25°C followed by 38 and 12°C. Lastly, *Lentimicrobium* abundances decreased from 12 to 25°C and were negligible in all replicates at 38°C and above.

Community Compositions at 45 and 60°C

At 45°C, the community composition was markedly different to that observed at 12, 25, and 38°C. Most replicates showed only one period of active mineralization, which occurred within the first 7 days of incubation. For the days 4 and 7 communities, dominant phylotypes were related to Bacilli, Campylobacteria, and Spirochaetia. Unlike phase 1 replicates at 12, 25, and 38°C, Alphaproteobacteria were not considerably enriched, ranging between ∼2–10% on days 3, 4, 7, and 14. Instead, several putatively mesophilic/thermophilic aerobes from the Bacilli lineage were present such as *Aeribacillus* (∼84%) on day 3, *Bacillus* (64–76%) on days 4 and 7 and *Brevibacillus* (∼28%) on day 14, indicating favorable temperature and electron acceptor condition. Furthermore, an uncultured Spirochaetia member (4–46%) and *Sulfurovum* (∼2–11%) were also present between days 3 and 14. The single replicate at 45°C (i.e., 45°C-3) which showed a second sulfidogenic phase was sacrificed after acetate was completely mineralized. The abundance of Bacilli was significantly lower (∼8.6%); specifically, sequences of the genera *Bacillus* (∼3%) and *Brevibacillus* (∼5%) which were less abundant when compared to the replicates on days 3–14. Instead, members belonging to Anaerolineae, Campylobacteria and Spirochaetiea emerged as the dominant class phylotypes. On the genus level, these are the *Pelolinea* (∼14%), uncultured Anaerolineaceae (∼13%), an uncultured Spirochaetaceae member (∼32%), and *Sulfurovum* (∼9%). *Desulfatiglans* had a relatively low but stable abundance throughout the 45°C incubations (∼1–3%), whereas other known sulfate-reducers were below 1%.

The samples incubated at 60°C which showed only one mineralization phase were sacrificed on days 4 and 7. At the class level, the community composition was dominated by Bacilli (∼32–88%). At the genera level, differences were observed amongst the replicates. Amongst the Bacilli, dominant genera were *Brevibacillus* (∼43%) on day 4 and *Planifilum* (∼89%) on day 7. In the day 4 replicate, *Pelolinea* (∼5%), *Desulfatiglans* (∼6%), *Desulfobacca* (∼3%) were also present but decreased at day 7 to low abundances (<1%).

Succession of Communities Caused by Temperature

The Bray–Curtis Dissimilarity analysis of the microbial community data shows a distinct difference between temperatures and phases represented as three main clusters (**Figure 6**). The first cluster (indicated by filled blue, yellow or green spheres) represents communities incubated at 12, 25, or 38°C corresponding to the aerobic phase 1 (**Table 1**).

The second cluster (indicated by hollow blue, green, and yellow spheres) comprised microcosms incubated at 12, 25, and 38°C sacrificed after day 36 (denoted as > 36), and including day 36 (indicated by filled squares in green and yellow). Cluster 2 includes communities sacrificed at a later incubation time point at which considerably more acetate (53–94%) had been mineralized compared to the communities in cluster 1. The third cluster (indicated by filled or hollow orange or red spheres and black asterisks) is made of the microbial communities incubated at 45 and 60°C, and communities from the inoculum (**Figure 1** and **Table 1**). Microcosms from 45°C (except replicate 45°C-3) and 60°C presented in this cluster were all sacrificed within the first 14 days, with a maximum mineralization of 21%. Additionally, this cluster also includes the sole active replicate 45°C-3, which achieved 98% mineralization after 190 days. The third cluster is also different from clusters 1 and 2. Community compositions between microcosms incubated at 12–25–38°C (cluster 1) were different from the inoculum (cluster 3), but the latter was more similar to the setups at 45–60°C (Cluster 3). Notably, communities at 45 and 60°C were likely different to those incubated at 12, 25, and 38°C. Secondly, two significantly different communities were established in the 12–25–38°C setting, belonging to phases 1 (cluster 1) and 2 (cluster 2) which suggests significant microbial community shifts with incubation times.

DISCUSSION

The investigated microbial community was originally enriched from a sulfidogenic, hydrocarbon-contaminated aquifer, and shown to be diverse and capable of degrading several aromatic hydrocarbons (Kleinsteuber et al., 2008; Herrmann et al., 2010; Taubert et al., 2012). Acetate was shown to be a metabolite upon sulfidogenic benzene mineralization, putatively metabolized by various phylogenetically different organisms (Rakoczy et al., 2011; Starke et al., 2016). We used this community as a model to determine the temperature range of anaerobic acetate mineralization and study the potential acetate-utilizing sulfate reducing degraders at different temperatures.

We postulate that mineralization occurred in two phases, a first aerobic phase and a second sulfidogenic phase. Aerobic mineralization is indicated by (i) the lack of sulfide (**Supplementary Figure S2**) or methane production from the reduction of sulfate and bicarbonate, the two principal electron acceptors present during the experimental set-up, and (ii) the bloom of typical aerobes affiliated to Alphaproteobacteria belonging to the genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (ANPR) (at 12–38°C) or Bacilli (at 45 and 60°C) (**Figure 4**). We rule out that nitrate or ferric iron might have been used as electron acceptors in this study, as both compounds were not amended. The coarse sand contains iron, but reduction of ferric iron has never been observed in former studies using this material (Vogt et al., 2007; Herrmann et al., 2008, 2010; Kleinsteuber et al., 2008; Taubert et al., 2012). Our data show a succession from oxic to anoxic conditions, illustrating conditions which usually occur in shallow subsurface systems and which could be promoted by ATES by introducing oxic heated or cooled water, thereby allowing us to compare mineralization rates of acetate under oxic and sulfate-reducing conditions in an altering microbial community. Given that acetate-degraders are ubiquitous since acetate occurs as a key intermediate in both aerobic/anoxic systems (Thauer et al., 1989), studying temperature or electron acceptor related shifts are therefore essential. Other studies focusing on communities' behavior and successions at permanent anoxic conditions under different heat-change regimes are in progress (data not shown) and will be published separately. Approximately 0.2 mM acetate (equivalent to ∼20% of the amended amount) was consumed in the first phase, implying that up to 0.4 mM oxygen was supposedly available (**Table 1**). Under oxic conditions, acetate oxidation to carbon dioxide with oxygen ($\Delta G_r^\circ = -908$ kJ mol^{-1}) is far more exergonic, outcompeting other electron acceptor such as sulfate ($\Delta G_r^\circ = -63$ kJ mol^{-1}) (Thauer et al., 1989; Jesußek et al., 2013). However, once oxygen was depleted, the dominant aerobic phylotypes were replaced by communities consisting mainly of anaerobic phylotypes such as Deltaproteobacteria, Ignavibacteria and Anaerolineae.

The ANPR taxon encompasses a wide range of species (Berrada and Fikri-Benbrahim, 2014), aerobes or facultative anaerobes (mainly utilizing nitrates). Most phylotypes are mesophilic (Berrada and Fikri-Benbrahim, 2014), such as the recently isolated *R. terrae* sp. *nov*, exhibiting growth between 0–42°C (Ruan et al., 2020); accordingly, the immediate acetate-dependent growth of these putative aerobic phylotypes at 12, 25, and 38°C indicates a broad mesophilic temperature range of members of this genus. Besides ANPR, *Thiobacillus*-like members were also thriving in the initial aerobic phase at 12°C, 25°C, and especially at 38°C, likely from the aerobic oxidation of iron sulfides which have been previously identified in the used coarse sand (Vogt et al., 2007). These form black precipitates visible to the eye, and may be supported by acetate which is used as a carbon source by some *Thiobacilli* species (Robertson and Kuenen, 2006).

The Bacilli which became enriched in phase 1 at 45 and 60°C, belonging to the genera *Bacillus*, *Aeribacillus*, *Brevibacillus*, and *Planifilum* are affiliated to typical thermophiles. Some bacilli are capable of growth at 50°C (*B. subtilis*) and 65°C (*B. stearothermophilus,* range: 30–75°C), as well as on anaerobic agars at 50°C (e.g., *B. licheniformis*, *B. laterosporus*); and many can form resistant endospores (Warth, 1978; Slepecky and Hemphill, 2006). *Aeribacillus*, represented by only two species recently, *A. pallidus* and *A. composti*, has been described as thermophilic, aerobic, endospore-forming bacteria which can utilize acetate (Yasawong et al., 2011; Filippidou et al., 2015; Finore et al., 2017). *Brevibacillus* isolates (e.g., *B. borstelensis*) were reported to grow in the range of 40–70°C (Panda et al., 2014; Khalil et al., 2018). Also, *Planifilum* species were described as thermophiles (Hatayama et al., 2005; Zhang et al., 2007). Generally, formation of endospores allows survival at lower temperatures in the absence of oxygen; hence, the immediate endospore reactivation at higher temperatures explains the quick bloom of aerobic thermophiles at 45 and 60°C (Keynan et al., 1964; Berg and Sandine, 1970). The low observed mineralization of acetate at 80°C in one replicate is possibly due to weak activity of thermophiles thriving at maximal but lethal incubation temperature; indeed, the data indicate that hyperthermophilic phylotypes were absent or below detection limit in the microbial community investigated. These findings demonstrate a resilience of acetate-mineralizing aerobes over a temperature range of 12–60°C stemming from an inoculum which was held at anoxic, sulfidic conditions for several years and presumed to be dominated by obligate anaerobic phylotypes at the onset of the experiment (**Figures 3**, **4**). Furthermore, the established aerobic communities are distinct between 12–38°C and 45–60°C.

Once oxygen was depleted, the second mineralization phase commenced following a period of lag phase and was more apparent (54–94% mineralized). Sulfide production peaked and stabilized between 0.8 and 1 mM for temperatures of 12, 25, and 38°C. Sulfide produced in Phase 2 was comparable to the theoretical sulfide production stoichiometry with respect to acetate mineralization, verifying that these two processes are connected (**Table 2**) and that sulfate was utilized as the terminal electron acceptor. The complete oxidation of 1 mM of acetate by dissimilatory sulfate reduction would yield 1 mM of sulfide and 2 mM of carbon dioxide (Eqn. 2). We calculated the ratio of sulfide produced to acetate mineralized for the second phase, discounting mineralization arising from the first aerobic phase (**Table 2**). Ratios of sulfide produced to acetate mineralized were < 1 at 12, 38, and 45°C-3, likely due to precipitation of FeS as previously described (Vogt et al., 2007; Herrmann et al., 2008; Kleinsteuber et al., 2008). Still, this relationship is within theoretical expectations (Kleikemper et al., 2002; Ozuolmez et al., 2015), including the value of 1.01 at 25°C (this study).

$$CH_3COO^- + SO_4^{2-} \rightarrow 2\,HCO_3^- + HS^- \quad (2)$$

In the 12–38°C setups, prolonged incubation saw putative sulfate reducing taxa becoming more evident including the genera *Desulfotomaculum* and *Desulfoprunum* (**Figure 5**), with the former seemingly more prevalent at 12 and 25°C, and not being represented at temperatures 38°C and above. *Desulfotomaculum* form endospores, and some species have been described for acetate oxidation (Widdel and Bak, 2006); psychrophilic *Desulfotomaculum* strains have been also previously reported (Klemps et al., 1985; Bahr et al., 2005; Aullo et al., 2013). Notably, mesophilic and moderately thermophilic *Desulfotomaculum* species were seemingly absent or below detection limit in our study although often described (Klemps et al., 1985; Scholten and Stams, 2000; Widdel and Bak, 2006; Aullo et al., 2013; Liu et al., 2018). For the genus *Desulfoprunum*, only a single species has been described so far, *D. benzolyticum*, a mesophilic freshwater isolate capable of acetate utilization (Junghare and Schink, 2015). While their study described an optimal growth of *D. benzolyticum* of 30°C with only little growth observed at 20 or 37°C, our results suggest that the *Desulfoprunum*-related phylotypes grown at 12°C are adapted to lower temperature ranges.

At 38°C, typical sulfate reducers also significantly increased in abundance in the sulfidic phase. For example, at day 36, these phylotypes comprised of *Desulfurispora* (11.6%) and *Desulfoprunum* (2.9%). Meanwhile, at day 122, *Desulfatiglans* (∼3.4%), *Desulfoprunum* (∼1.1%), *Desulforivibrio* (2.2%), and *Desulfurispora* (1.3%) were amongst the abundant sulfate reducers. It is worth to note that at day 36 acetate was still available, while these was completely utilized prior to sampling day 122, hence acetate unavailability could be a reason for the lower abundance, which could be attributed to other factors such as cell lysis or spore formation. The fact that *Desulfurispora* was highly abundant at day 36 hints that this organism metabolized acetate at 38°C. Notably, to our knowledge only one such species was described yet, growing at 40-67°C and unable to utilize acetate (Kaksonen et al., 2007). In contrast, the *Desulfurispora* phylotype seen in our study is abundant under mesophilic conditions, and as we did not detect any sulfate-reducing activity at 60°C. Lastly, *Desulfatiglans*, also detected in communities at 12 and 25°C have been reported for acetate oxidation under sulfate reducing conditions elsewhere (Jochum et al., 2018).

Besides the sulfate reducers, several other phylotypes also saw an uptick in abundance at 38°C. At day 36, phylotypes included an uncultured member of the Ignavibacteria (5.2%), *Sulfurovum* (6.5%), and uncultured member of the Anaerolineae (3.7%). Similarly, increased abundances were also reported at day 122, amongst them, an uncultured Anaerolinea member (5.1%), *Sulfurovum* (9.1%), three uncultured members of the Ignavibacteria (2.3–3.9%), which hinted at a possible role in acetate oxidation under mesophilic conditions. Ignavibacteria, with several members abundant in both 25 and 38°C setups, are not known for sulfate reduction, but were detected in various hydrocarbon-degrading microbial communities under various electron acceptor conditions including sulfate (Kummel et al., 2015; Westphal et al., 2017; Keller et al., 2018). However, their function in acetate metabolization at sulfate reducing conditions in our study is unclear. *Sulfurovum*, belonging to the Campylobacterales (Epsilonproteobacteria), was detected

in several replicates at 38°C. Interestingly, Campylobacterales, have been reported as potential acetate consumers at sulfate-reducing conditions detected by protein-stable isotope probing studies using uniformly ^{13}C-labeled acetate as substrate (Starke et al., 2016). In addition, Epsilonproteobacteria or specifically *Sulfurovum*-like phylotypes were enriched in hydrocarbon-degrading consortia (Kleinsteuber et al., 2008; Muller et al., 2009; Herrmann et al., 2010) which suggest that the *Sulfurovum* enriched in our 38°C setup could have consumed the acetate. While *Sulfurovum* was detected over a range of temperatures (22–66°C) in water-flooded oil reservoirs, they were not particularly enriched (Tian et al., 2017). However, in our study, *Sulfurovum* was particularly enriched at 38°C (up to 9%) and 45°C (up to ∼11%) while abundances were lower at 12°C (up to ∼4%) and 25°C (∼2%). In contrast, *Sulfurovum* abundance was negligible in almost all replicates at 60°C (<1%) which did not mineralize acetate at sulfate-reducing conditions. Hence, this Sulfurovum candidate might be active within the higher end of the mesophilic range (38–45°C).

One key result of our study is the observation that temperatures above 38°C (45°C, 60°C) strongly inhibited acetate mineralization at anoxic conditions, as only one replicate at 45°C could do so. Notably, none of the typical sulfate reducers that became enriched at 12–38°C seem to be abundant in this replicate, which raises the question of which phylotypes is actually responsible for sulfate reduction. The Spirochaetes, which increased to up to 32%, are often observed at anoxic hydrocarbon-contaminated sites; they are suggested to recycle necromass by fermentation, and have not been described to be directly involved in hydrocarbon degradation at anoxic conditions (Dong et al., 2018). Our data indicate that Spirochaetes are especially adapted to temperatures above the typical mesophilic range, which seem to be true as well for members of the Anaerolineaceae whose abundance increased to up to 27%. Anaerolineae were shown to comprise thermophilic members and were reported to reach high abundances during anaerobic digestion under methanogenic or sulfate reducing conditions, but due to a general lack of isolates and genomes their physiological capabilities are less well understood (Xia et al., 2016; Roy et al., 2018). Hence, it might be that these phylotypes become enriched by scavenging on dead biomass similar to the Spirochaetes. Although not yet described for dissimilatory sulfate reduction, we cannot rule out the possibility that some of them are able to do so. For instance, in a down-flow fluidized bed reactor operated at 25°C and mainly fed with acetate and sulfate, a community developed which comprised of 50% of Anaerolineaea and to a lesser extent of typical acetate-oxidizing sulfate reducers (Montoya et al., 2013). Recently, Anaerolineae phylotypes were shown to assimilate ^{13}C-labeled acetate upon syntrophic acetate oxidation in a chemostat under methanogenic conditions (Yi et al., 2020); thus, it is also possible that acetate was syntrophically oxidized in our culture by Anaerolineae phylotypes, leaving the question open which organisms were actually responsible for sulfate reduction. Also 45°C lies within the overlap of the maxima end of the mesophilic range and the minima end of the thermophilic range, in the so called mesophilic-thermophilic boundary. Not much is known of bacteria tolerant in this boundary. Notably, in other studies, sulfide production was observed at higher temperatures compared to our study (Elsgaard et al., 1994; Bonte et al., 2013; Jesußek et al., 2013; Westphal et al., 2017).

Three distinct clusters in the Bray–Curtis confirmed the described shift in the microbial communities at 12, 25, and 38°C in phase 1 (cluster 1) to phase 2 (cluster 2), which are both markedly different from the inoculum (cluster 3). Community shifts are usually observed in environments shifting from oxic to anoxic conditions (Broman et al., 2017; Bush et al., 2017). Additionally, samples from replicates at 45 and 60°C in phase 1 contained sequences belonging to cluster 3 and were thus similar to the inoculum; the main difference between them is the emergence of putative aerobic species such as the Bacilli (**Figure 5**). The sulfidogenic replicate 45°C-3 also grouped in Cluster 3, although having a lower abundance of Bacilli-like phylotypes which were replaced by higher Anaerolineae and Spirochaetia abundances. The composition of cluster 3 of the Bray–Curtis analysis indicates that only a few members of the inoculum were actually active at temperatures $\geq$ 45°C. Notably, the results of our study are in contrast to those reported by Westphal et al. (2017) who observed acetate mineralization under both oxic and sulfate-reducing conditions in aquifer sediment columns experiments at 10, 25, 40, and 70°C.

The temperature growth/biochemical reaction correlation (Davey, 1993) would imply that mineralization rates increase with temperature (Meier et al., 2005; EPA, 2017). Similarly, increased rates in organic matter mineralization between 25 and 50°C were also reported (Hossain et al., 2017). However, in our study, this was not necessarily the case. In the aerobic phase, two temperature maxima were observed, first at 25°C (75 ± 34.4 μM d^{-1}) and second at 60°C (71.4 ± 24.3 μM d^{-1}). Mineralization rates increased from 12 to 25°C, in agreement with increased mineralization from 6 to 28°C observed before (Sansone and Martens, 1982). However, rates at 38, 45, and 60°C did not fit with this relationship, given that rates at 25 and 38°C (74.1 ± 20.2 μM d^{-1}) were similar; and a rate minimum was observed at 45°C, being about six times lower than the values at 25 and 38°C. In the anaerobic phase, considering only replicates at 12–25–38°C, acetate mineralization rates (9.8 ± 4.3, 18.8 ± 1.5, 22.9 ± 5.3 μM d^{-1}) matched the Arrhenius relationship, increasing with temperature (**Table 1**). The increased mineralization activity at 25 and 38°C compared to 12°C corroborates with prior studies on the enhanced biodegradation with increased temperatures (Jesußek et al., 2012; Griebler et al., 2016; Westphal et al., 2017). In contrast, the single sulfidogenic replicate 45°C-3, does not fit with the linearity observation, indicating non-optimal conditions for the acetate consumers at 45°C as microbial growth rates outside optimal conditions decrease with temperature until inhibition (Mohr and Krawiec, 1980; Okabe and Characklis, 1992; Huang et al., 2011). Inhibition of degradation processes at temperatures above 45°C has also been shown in other studies, e.g., for dechlorination (Deeb and Alvarez-Cohen, 1999;

Friis et al., 2007b; Fletcher et al., 2011). Clearly, more studies are needed using different types of sediments to derive general assumptions on the physiological capacities of indigenous microbial communities at different, especially high ($T \geq 45°C$) temperatures; inhibition temperatures might be experiment- or site-specific.

Implications and Outlook

Our study highlights the key role of temperature toward microbial degradation at both oxic and strictly anoxic conditions. Albeit limited to laboratory batch microcosms, our study provides data on fundamental microbial community shifts and activities which help to evaluate related processes in real ATES systems. Our data emphasize the importance of the interplay of electron acceptor presence and temperature toward the activities of single members of the microbial community, and provides indications for possible temperature limits at distinct redox conditions. From the standpoint of ATES in temperate zones, with ambient groundwater temperatures of ca. 8–14°C, rates are expected to be higher with increasing temperature. At temperatures up to 38°C, which is beyond that of the typical ATES temperature threshold of 25°C in many European countries, sustained mineralization rates were observed at both oxic and anoxic conditions. However, the ultimate aim remains to assess the feasibility of HT-ATES, operating at temperature conditions only favorable for thermophilic or hyperthermophilic species. Meanwhile, the mesophilic-thermophilic boundary, such as 45°C could be an interesting starting point for future studies when trying to combine HT-ATES and *in situ* bioremediation, by identifying and characterizing mesophilic/thermophilic species capable of contaminant degradation. With observed sulfide production at 45°C, the feasibility for such reactions to occur at this temperature remains open; clearly more studies are required to understand the community resilience in the mesophilic-thermophilic boundary, using sediments of different origin and history.

In this study, temperature thresholds for aerobic mineralization are found to be higher compared to mineralization at sulfate-reducing conditions. This becomes the main caveat in the subsurface that become rapidly void of oxygen, particularly in contaminated zones (EPA, 2013). While aerobic mineralization is feasible (up to 60°C-this study) this cannot be assumed for anoxic condition. Nevertheless, HT-ATES could largely benefit from the twofer outcomes that allow higher thermal energy storage whilst simultaneously remediating contaminants without compromising anaerobic mineralization processes and rates. The uncertainty on how the combined heat storage and bioremediation approach could be achieved at elevated temperatures, i.e., >45°C, in bioremediation of hydrocarbon-contaminated aquifers still needs to be addressed. Additionally, seasonal ATES operations would drive subsurface temperature fluctuations (Saito et al., 2016), more so in HT-ATES. A deeper understanding of the effects of thermal energy storage and extraction toward mineralization and microbial community is an important step toward the goal of raising thermal storage thresholds. Community resilience and thermal cycling fatigue could also threaten ATES performance. Heat cycling experiments, by combining different heat exchange scenarios, together with stable isotope probing could elucidate the long-term effects of HT-ATES on ecosystem services and bioremediation potential. Furthermore, repopulation of HT-ATES from aquifer recharge or bioaugmentation could sustain or enhance bioremediation at elevated temperatures (Friis et al., 2007a,c).

Summary

A microbial community adapted to sulfate reduction and hydrocarbon degradation amended with acetate showed distinct temperature-related effects on mineralization and sulfide production pattern observed at temperatures up to 38°C. Sulfide production was notably absent at 60°C and 80°C, and in two of three replicates at 45°C. Biphasic mineralization phases at 12°C to 38°C and a single phase mineralization at 60°C illustrated the consecutive use of oxygen and sulfate as electron acceptors at 12–38°C and the exclusive use of oxygen as acceptor at 60°C. At 80°C, aerobic mineralization was likely impaired while mineralization under sulfate reduction was absent, likely from the absence of thermophiles. Microbial community analysis as well as Bray–Curtis dissimilarity further highlights the role of temperature in community differences under aerobic and sulfate-reducing environments. The discovery of aerobic Alphaproteobacteria from an anaerobically maintained inoculum, suggests a notable resilience of these aerobes living under prolonged anoxic conditions. Meanwhile, Bacilli detected at the mesophilic/thermophilic range would be of no surprise owing to its endospore-forming ability. Aerobic acetate mineralization rates were comparable at 25, 38, and 60°C but about six to seven folds higher than at 12 and 45°C. Anaerobically, acetate mineralization rates for 12, 25, and 38°C increased with temperature, with stoichiometric production of sulfide, however, the lowest rate was observed in the sole sulfide producing replicate at 45°C. Microbial community analysis only found several typical sulfate reducers such as *Desulfoprunum* with varying abundances at 12 to 38°C. In contrast, no typical sulfate-reducers were significantly stimulated in the sulfidogenic 45°C replicate; instead Anaerolinea and Spirochaetia members were more dominant. Herein, this study found that temperatures up to 38°C does not seem detrimental to the removal of acetate, but a higher temperature (>45°C) remains a limitation where remediation strategies are concerned. Nevertheless, this proposes a potential new discussion for sulfate reduction at the mesophilic-thermophilic boundaries especially if HT-ATES proved unfeasible. This study demonstrates how the community could adapt to temperature and electron acceptor availability. More importantly, temperature could influence mineralization rates, as well the potential actors involved in degradation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB40338.

AUTHOR CONTRIBUTIONS

MB planned and performed the experiments, analyzed the data, and wrote the manuscript. CV planned the experiments and edited the manuscript. HR edited the manuscript. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.606565/full#supplementary-material

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Chapter 3.

Sulfidic acetate mineralization at 45°C by an aquifer microbial community: key players and effects of heat changes on activity and community structure

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Sulfidic acetate mineralization at 45°C by an aquifer microbial community: key players and effects of heat changes on activity and community structure

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Summary

High-Temperature Aquifer Thermal Energy Storage (HT-ATES) is a sustainable approach for integrating thermal energy from various sources into complex energy systems. Temperatures ≥45°C, which are relevant in impact zones of HT-ATES systems, may dramatically influence the structure and activities of indigenous aquifer microbial communities. Here, we characterized an acetate-mineralizing, sulfate-reducing microbial community derived from an aquifer and adapted to 45°C. Acetate mineralization was strongly inhibited at temperatures ≤25°C and 60°C. Prolonged incubation at 12°C and 25°C resulted in acetate mineralization recovery after 40–80 days whereas acetate was not mineralized at 60°C within 100 days. Cultures pre-grown at 45°C and inhibited for 28 days by incubation at 12°C, 25°C, or 60°C recovered quickly after changing the temperature back to 45°C. Phylotypes affiliated to the order *Spirochaetales* and to endospore-forming sulfate reducers of the order *Clostridiales* were highly abundant in microcosms being active at 45°C highlighting their key role. In summary, prolonged incubation at 45°C resulted in active microbial communities mainly consisting of organisms adapted to temperatures between the typical temperature range of mesophiles and thermophiles and being resilient to temporary heat changes.

Introduction

A considerable part of fossil fuels is globally consumed for heating and cooling purposes (Elsland *et al*., 2017; Heatroadmapeurope, 2019). Aquifer Thermal Energy Storage (ATES) promises a more sustainable supply of thermal energy by storing and extracting heat in the subsurface. In addition to storing thermal energy from heat-generating industries such as incineration plants, ATES can operate in seasonal mode, by storing excess thermal energy in aquifers during the summer, which can then be used during the cooler months. More than 90% of ATES in operation worldwide are located in Europe (Fleuchaus *et al*., 2018). A majority of the ATES in operation are Low-Temperature (LT-ATES) installations, which function under an upper limit temperature threshold (e.g. 25°C). However, by operation of High-Temperature ATES (HT-ATES) systems with storage temperatures >40°C, an integration of (non-)renewable heat sources into complex energy systems is possible (Fleuchaus *et al*., 2020). Some of the limitations that contribute to the lower adoption of HT-ATES largely stem from long-term operational costs, uncertainty on their environmental impact, and regulation issues (Fleuchaus *et al*., 2018; Fleuchaus *et al*., 2020).

ATES is especially attractive for urban or industrial areas due to its high energy production and consumption. Aquifers in those sites are often contaminated by organic pollutants (National Research Council, 2000), and combining remediation techniques with ATES is an attractive approach. As groundwater temperatures within the ATES core zone and vicinity are affected, this will influence a contaminant's physicochemical characteristics and its attenuation potential. Petroleum hydrocarbons constitute the most common contaminants introduced into the subsurface through leakage or improper disposal (Rooney-Varga *et al*., 1999; Da Silva and Alvarez, 2004; Vogt *et al*., 2007). Both biotic and abiotic attenuation of contaminants can occur, but the latter contribution tends to be limited in aquifers, where oxygen is rapidly depleted (USEPA, 1999; Meckenstock *et al*., 2015). During syntrophic hydrocarbon degradation, acetate is a commonly occurring intermediate in the several pathways reported (Jones *et al*., 2008; Gieg *et al*., 2010; Fuchs, 2011; Lueders, 2017). In the presence of terminal electron acceptors, acetate can serve both as carbon source

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and electron donor becoming mineralized to carbon dioxide (Wu *et al*., 1997; Ho *et al*., 2002; Heuer *et al*., 2010; Head *et al*., 2014). Due to the availability of sulfate in various ecosystems, dissimilatory sulfate reduction is among the most commonly described metabolism under anoxic conditions (National Research Council, 2000; Miao *et al*., 2012).

In an ecosystem comprising a plethora of microorganisms, microbial processes are not as straightforward as correlating biotransformation with the Arrhenius equation (Garcia *et al*., 2018). The growth temperature, which is an intrinsic characteristic of each species, is defined by the maxima, minima and optima, which connect microbial growth to temperature (Dettmer, 2002). Maximal growth rates can be achieved around the optimal temperature, whereas at higher temperatures rates rapidly decrease and cease under sub-optimal conditions (Mohr and Krawiec, 1980; Huang *et al*., 2011). For instance, this can be observed with the different microbial growth ranges in psychrophiles (0–20°C), mesophiles (8–48°C), thermophiles (40–70°C) and hyperthermophiles (65–90°C) (Madigan *et al*., 2017). Heterogeneity and localized niches can also affect attenuation processes (Tian *et al*., 2017; Garcia *et al*., 2018; Pannekens *et al*., 2019). The effects of temperature are thus multi-faceted, since temperature does not just affect physicochemical characteristics of the contaminants (Koproch *et al*., 2019), but it can also affect the microbial community and the biotransformation processes (Dettmer, 2002; Higashioka *et al*., 2011). Temperature fluctuations arising from injection and extraction of water into aquifers could largely influence the general activity of the microbial community as well the ability to mineralize distinct contaminants (Hossain *et al*., 2017).

In a previous study (Bin Hudari *et al*., 2020), using a hydrocarbon-degrading microbial community spiked with uniformly ^{13}C-labelled acetate under sulfate reducing condition, acetate mineralization was observed at 12°C, 25°C and 38°C, whereas acetate was not mineralized at 60°C and 80°C, while only one of three replicates incubated at 45°C mineralized acetate with concomitant sulfide production. In that study, 45°C was selected, because it lies in the overlap of mesophilic-thermophilic growth boundary (Madigan *et al*., 2017), which could potentially harbor a niche group of heat-tolerant microorganisms. In an HT-ATES system, 45°C can be found either in the core zone or in the outer impact zone (Sanner *et al*., 2005; Lerm *et al*., 2013; Kallesøe and Vangkilde-Pedersen, 2019). This could also influence the surrounding zone which is in close proximity to the core by indirectly enhancing bioremediation activity (Todorov *et al*., 2020). If bioremediation activity can be sustained at this temperature (45°C), this would be an extra advantage in addition to allowing a greater thermal energy capacity with the implementation of HT-ATES. While there have been studies to assess the likely impacts of temperature in ATES (Jesußek *et al*., 2012; Jesußek *et al*., 2013; Ni *et al*., 2015) as well as in other applications (Deeb and Alvarez-Cohen, 1999; Friis *et al*., 2007a,b,c; Yadav *et al*., 2012; Zeman *et al*., 2014), studies on the effects of periodic heat storage subject to different temperature exposure on biotransformation are limited. Therefore, our microcosm-based study aims to explore the effects of periodic heat exchange on acetate mineralization, sulfide production, their rates and microbial community composition. Starting with an inoculum comprising an aquifer microbial community enriched at 45°C, microcosms were subjected to different treatment regimes within three scenarios: (i) 'control' at 45°C, (ii) 'adaptation' from 45°C to 12°C, 25°C, or 60°C and incubated for up to 133 days and (iii) 'heat exchange' scenario where microcosms were temporarily exposed to 12°C, 25°C, 38°C, or 60°C for a 28-day period before switching again to 45°C.

Results

Activities at 45°C and after subsequent permanent temperature shifts to 12°C, 25°C or 60°C

These experiments were done to determine general activity parameter at 45°C, to characterize the activities of the 45°C-adapted community at lower and higher temperatures ($\Delta T \geq 15$°C) and to determine the resilience of the community to mineralize acetate at lower and higher temperatures after prolonged incubation. In microcosms incubated continuously at 45°C (treatment 1), acetate was mineralized in all three replicates with concomitant sulfide production and no apparent lag phase (Fig. 1A and B). Within the first 6.25 days, acetate was mineralized at an average rate of 52.9 $\pm$ 8.5 μM d^{-1} while sulfide was produced at an average rate of 51.8 $\pm$ 3.8 μM d^{-1}. Subsequently, both rates increased to 85.4 $\pm$ 17.3 (mineralization) and 65.7 $\pm$ 2.9 μM d^{-1} (sulfide production), indicating increasing cell numbers by growth. After 10 days of incubation, 87.5 $\pm$ 17.7% (875.4 $\pm$ 177 μM) of the added acetate was mineralized accompanied by production of 657.4 $\pm$ 29 μM sulfide (Tables 1 and S3).

In treatments 2–4 of the 'adaptation scenario', average acetate mineralization and sulfide production rates during the 'Pre' stage at 45°C were comparable to treatment 1 values (Table 1). Transferring the communities to either 12°C, 25°C or 60°C resulted in significantly changing mineralization and sulfide production rates; generally, the selected temperatures caused an immediate inhibition of activity. A temperature of 12°C (treatment 2, 45 $\rightarrow$ 12°C) completely inhibited acetate mineralization for around 63 days; thereafter, only two of three replicates mineralized acetate at a rate of 13.9 $\pm$ 1.4 μM d^{-1} and produced sulfide at 11.6 $\pm$ 1.7 μM d^{-1} (Table 1, Fig. 1C and D, Table S3).

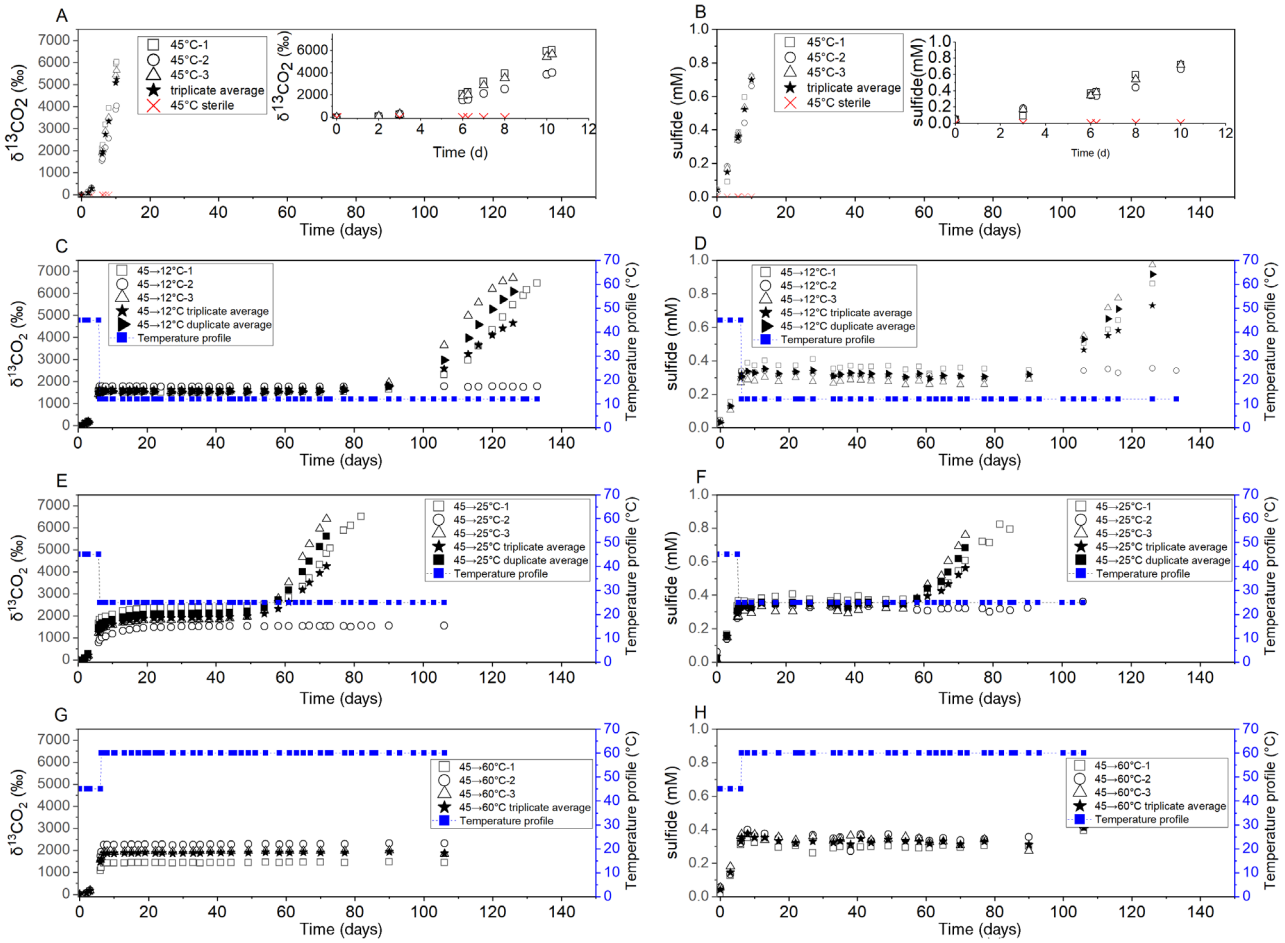


Fig. 1. Time courses development of ^{13}C-labelled CO_2 and sulfide for treatments 1 (45°C) (A, B), 2 (45 → 12°C) (C, D), 3 (45 → 25°C) (E, F) and 4 (45 → 60°C) (G, H) respectively. Additional insert in diagrams in A and B represent the data for the first twelve days of incubation at higher resolution. Data are presented for each respective replicate and the corresponding average of these replicates.

Shifting the temperature to 25°C (treatment 3, 45 → 25°C) did not completely inhibit acetate mineralization as observed for 12°C, but rates were tremendously decreased (Table 1, Fig. 1E and F). After more than 50 days incubation at 25°C ('End' stage), acetate mineralization and sulfide production started to increase again in two of three replicates at rates of 11.3 ± 1.7 and 6.7 ± 0.9 μM d^{-1} respectively (Table 1, Fig. 1E and F). The third replicate was excluded as mineralization ceased after a few days at 25°C (Fig. 1E, Table S3). Transferring the communities to a temperature of 60°C (treatment 4, 45 → 60°C) completely inhibited mineralization and sulfide production after some residual activity within the immediate 0.75 days after shifting to 60°C (Table 1, Fig. 1G and H; Table S3).

Activities at 45°C after temporary temperature shifts to 12°C, 25°C, 38°C or 60°C

These experiments were done to determine the resilience of the 45°C-adapted community to temporary lower or higher temperatures ($\Delta T \geq 7$°C). In the heat exchange setups (treatments 5–8), communities were first incubated at 45°C for 6 days ('Pre' stage), and then transferred to 12°C (treatment 5), 25°C (treatment 6), 38°C (treatment 7) or 60°C (treatment 8) for up to 28 days. On day 35, they were returned to 45°C ('Post' stage). In the 'Pre' stage, all replicates mineralized acetate and produced sulfide without lag-phase in similar rates as described for the permanent temperature shift experiments (Tables 1 and 2; Figs 1A and B, 2A–D, and 3A–D).

When replicates were transferred to the respective incubation temperature after 6 days ('Post' stage), acetate mineralization and sulfide production rates varied depending on the selected new temperature; in general, cultures behaved similarly to those observed for treatments 2–4. During the 29-day incubation at 12°C, acetate mineralization was not observed (treatment 5, Fig. 2A) while some residual activity was observed at 60°C, shortly after temperature change (5.0 ± 0.2 μM,

Table 1. Overview on results of triplicates averages of acetate mineralization, sulfide production and rates for treatments 1–4.

Objective	Control		Adaptation			
Treatment	1		Treatment	2	3	4
Batch	45°C	Stage	Batch	45 → 12°C	45 → 25°C	45 → 60°C
Duration(d)	6.25	Pre	Duration (d)	6.25	6.25	6.25
Acetate mineralized (%)	33.1 ± 5.3		Acetate mineralized (%)	27.4 ± 2.2	23.0 ± 7.6	27.1 ± 6.0
Acetate mineralized (μM)	330.9 ± 53.4		Acetate mineralized (μM)	274.2 ± 22.2	230.3 ± 76.0	270.8 ± 59.8
Rate at $t = 6.25$ (μM acetate d^{-1})	52.9 ± 8.5		Rate at $t = 6.25$ (μM acetate d^{-1})	43.8 ± 3.5	36.8 ± 12.2	43.3 ± 9.6
Duration (d)	6.25		Duration (d)	6.25	6.25	6.25
Sulfide produced (μM)	323.9 ± 23.6		Sulfide produced (μM)	292.3 ± 2.4	279.0 ± 56.0	313.1 ± 7.4 ($n = 3$)
Rate at $t = 6.25$ (μM sulfide d^{-1})	51.8 ± 3.8		Rate (μM d^{-1})	47.4 ± 1.0	44.6 ± 9.0	50.1 ± 1.2 ($n = 3$)
Duration (d)	10.25	Post	Duration (d)	56–63	31.75–75.75	75.75[a.]
Acetate mineralized (%)	87.5 ± 17.7		Acetate mineralized (%)	82.3 ± 1.6 ($n = 2$)	56.4 ± 40.1 ($n = 3$)	4.3 ± 1.0[a., c.]
Acetate mineralized (μM)	875.4 ± 176.9				79.5 ± 4.3 ($n = 2$)	
Rate (μM acetate d^{-1})	85.4 ± 17.3		Lag phase for acetate mineralization after temperature shift based on $\delta^{13}CO_2$ (d)	63.75	Not observed[b.]	-
Duration (d)	10		Acetate mineralized (μM)	822.7 ± 16.1 ($n = 2$)	564.5 ± 401.0 ($n = 3$)	42.8 ± 10.4
Sulfide produced (μM)	657.4 ± 29.0				795.4 ± 43.2 ($n = 2$)	
Rate (μM sulfide d^{-1})	65.7 ± 2.9		Rate (μM acetate d^{-1})	13.9 ± 1.45 ($n = 2$)	8.6 ± 4.8 ($n = 3$)	-[a.]
					11.3 ± 1.7 ($n = 2$)	
Sulfide produced/acetate mineralized (end of incubation)	0.77 ± 0.1		Duration (d)	56 & 63 ($n = 2$)	65.75 & 75.75 ($n = 2$)[d.]	-
			Sulfide produced (μM)	685.5 ± 41.2 ($n = 2$)	471.4 ± 19.3 ($n = 2$)[d.]	-
			Rate (μM d^{-1})	11.6 ± 1.7 ($n = 2$)	6.7 ± 0.9 ($n = 2$)[d.]	-[a.]

[a.] Acetate mineralization or sulfide production ceased, therefore corresponding rates were not calculated.
[b.] Mineralization was continuous but proceeded at low rate initially when transferred to 25°C, rates increased after longer incubation at 25°C (see Fig. 1E).
[c.] Acetate mineralization or sulfide production due to residual activity after shifting to 60°C.
[d.] Third replicate not considered, information in Table S3.

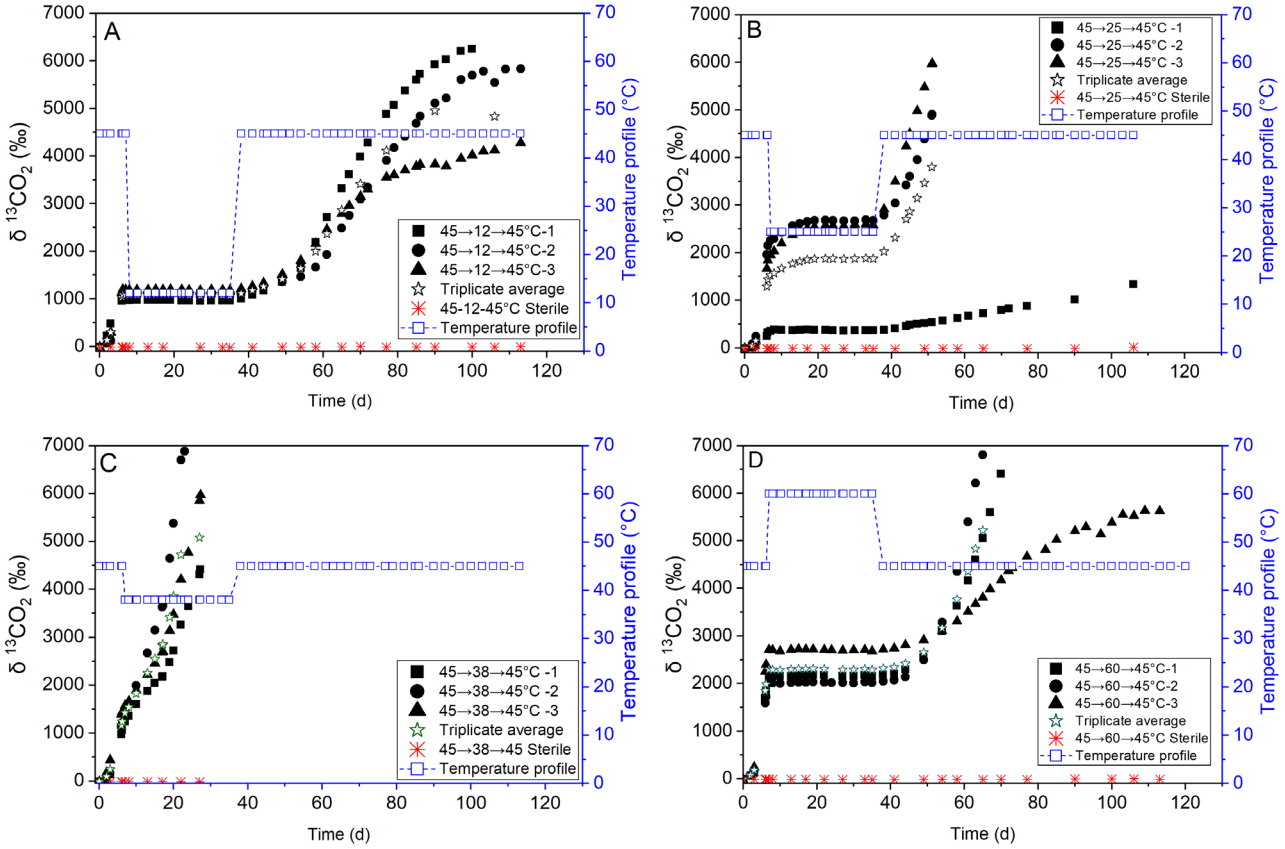


Fig. 2. Time courses of the development of ^{13}C-labelled CO_2 production from microcosms in treatments 5 (45° → 12 → 45°C) (A), 6 (45 → 25 → 45°C) (B), 7 (45 → 38°C) (C) and 8 (45 → 60 → 45°C) (D). In treatment 7, the experiment was completed during incubation at 38°C and was not returned to 45°C.

treatment 8, Fig. 2A). Sulfide production was not observed at both temperatures (Fig. 3A and D; Table S3). At 25°C (treatment 6), acetate mineralization phased out after 14 days and was afterward absent, with only 9.6 ± 8.3 μM (n = 3) acetate mineralized and similar small amounts of sulfide produced (Figs 2B and 3B, Table S3). In contrast, during incubation at 38°C (treatment 7), mineralization continued with no lag phase and was completed 16–21 days after shifting to the new temperature, and hence was not returned to 45°C (Fig. 2C, Table 2). Acetate was mineralized (742.4 ± 189.3 μM) at a rate of 39.1 ± 15 μM d^{-1}, while an average of 449 ± 80.2 μM sulfide was produced at 22.4 ± 5.5 μM d^{-1} (Table 2). Both rates were lower with respect to the control which was continuously incubated at 45°C (treatment 1, Table 1). Acetate mineralization rates were initially slightly lower as observed for 45°C when the replicates were placed at 38°C; however, the rates increased again with prolonged incubation at 38°C (Fig. 2C), where rates during incubation at 38°C were comparable to rates before temperature change at 45°C (Table 2), indicating growth of the acetate-mineralizing community.

At t = 35 days, treatments 5 (12°C), 6 (25°C) and 8 (60°C) were returned to 45°C. Acetate mineralization resumed in all three treatments, albeit at different rates (Fig. 2A, B, and D), alongside sulfide production (Fig. 3A, B, and D). Based on $\delta^{13}CO_2$ values, lag phases were not evident in all three treatments (Table S3). In treatment 5 (12°C), triplicate communities mineralized 722.8 ± 189 μM acetate at 10 ± 3.4 μM d^{-1} when returned to 45°C, while 591.2 ± 127.3 μM of sulfide was produced at a rate of 8.2 ± 1.6 μM d^{-1} (Table 2); these rates are about three to four times lower when compared to rates before temperature change at t = 6.25 d, indicating a negative effect of 12°C on the 45°C-adapted community. For treatment 6 (45 → 25 → 45°C), on average, 332.4 ± 168 μM of acetate was mineralized at 17.9 ± 14.4 μM d^{-1} (n = 3) with no lag phase after microcosms were returned to 45°C. One replicate recovered very slowly, with only 2.2 μM d^{-1} acetate mineralized and 1.7 μM sulfide d^{-1} produced in 71 days. These rates were around ten times lower when compared to the other two replicates (Table S3). The average acetate mineralization and sulfide production rates at 45°C after the temporary incubation at 25°C were about two times lower

Table 2. Overview on results of triplicates averages of acetate mineralization, sulfide production and rates for treatments 5–8.

Objective		Heat exchange			
	Treatment	5	6	7	8
Stage	Batch	45 → 12 → 45°C	45 → 25 → 45°C	45 → 38°C[a.]	45 → 60 → 45°C
Pre	Duration (d)	6.25	6.25	6.25	6.25
	Acetate mineralized (%)	18.4 ± 1.8	24.2 ± 16.3 ($n = 3$) 33.4 ± 3.17 ($n = 2$)	20.9 ± 3.13	33.3 ± 6.02
	Acetate mineralized (μM)	183.6 ± 18.7	241.7 ± 163.2 ($n = 3$) 334.7 ± 37.1 ($n = 2$)	208.9 ± 31.4	333.4 ± 60.2
	Rate (μM d^{-1})	29.4 ± 3.0	38.7 ± 26.1[b.]	33.4 ± 5.0	53.3 ± 9.6
	Duration (d)	6.25	6.25	6.25	6.25
	Sulfide produced (μM)	230.7 ± 49.0	288.4 ± 78.1	245.9 ± 16.6	336.4 ± 48.5
	Rate (μM d^{-1})	36.9 ± 7.8	46.1 ± 12.5	39.3 ± 2.6	53.8 ± 7.8
Post	Duration (d)	28.75	28.75	16.75–21	28.75
	Acetate mineralized (%) cc	Not observed	9.6 ± 8.3 ($n = 3$) 13.9 ± 5.1 ($n = 2$)	74.2 ± 18.9	5.0 ± 0.2
	Acetate mineralized (μM)	Not observed	95.9 ± 83.0 ($n = 3$) 139.2 ± 20.6 ($n = 2$)	742.4 ± 189.3	50.4 ± 1.8[c.]
	Rate (μM acetate d^{-1})	Not observed	3.3 ± 2.9[b.]	39.1 ± 15.0	n/c
	Duration (d)	28.75	28.75	18.75–21	28.75
	Sulfide produced (μM)	Not observed	12.3 ± 3.7 ($n = 2$)	449.0 ± 80.2	Not observed
	Rate (μMsulfide d^{-1})	Not observed	0.4 ± 0.1 ($n = 2$)[e.]	22.4 ± 5.5	n/c
End	Duration (d)	65–78	16.25–71	-	30–74
	Lag phase of acetate mineralization after temperature shift based on $\delta^{13}CO_2$ values (d)	Not observed	Not observed	-	Not observed
	Acetate mineralized (%)	72.3 ± 18.9	33.2 ± 16.8	Completed prior	65.0 ± 15.6
	Acetate mineralized (μM)	722.8 ± 189.8	332.4 ± 168.5	-	649.2 ± 156.0
	Rate (μM acetate d^{-1})	10.0 ± 3.4	17.9 ± 14.4	Completed prior	17.4 ± 13.1[b.]
	Duration (d)	71–74	16.25 ($n = 2$)[e.]	Completed prior	30–71
	Sulfide produced (μM)	591.2 ± 127.3	282.6 ± 76.9 ($n = 2$)	-	453.9 ± 34.2
	Rate (μM sulfide d^{-1})	8.2 ± 1.6	17.4 ± 4.7 ($n = 2$)	Completed prior	11.7 ± 5.3

[a.] Acetate mineralization was completed during incubation at 38°C, no return to 45°C.
[b.] Only averaged rate of triplicates shown.
[c.] Acetate mineralization due to residual activity after shifting to 60°C.
[d.] Rate based on acetate mineralization duration.
[e.] Third replicate not considered, information in Table S3.
n/c, not considered.

when compared to the initial average rate before the temperature change at $t = 6.25$ d.

In treatment 8 (45 → 60 → 45°C), while no mineralization was observed during 60°C incubation, mineralization and sulfide production resumed with no apparent lag phase when returned to 45°C on day 35 (Figs 2D and 3D). Acetate was mineralized and sulfide produced after return to 45°C in rates of 17.4 ± 13.1 ($n = 3$) and 11.7 ± 5.3 μM d^{-1} ($n = 3$) respectively (Table 2). These rates were about three to four times lower compared to rates before temperature change (Table 2).

Microbial community composition

The Miseq sequencing data of the 18 samples produced 1 270 196 16S rRNA gene sequence reads with average reads of 70 556 ± 16 103 per sample, with minimum and maximum sequence reads of 46 807 and 109 242 respectively. The amplicon sequence variants (ASVs) varied between 38 and 135 shown by the rarefaction curve of the unrarefied samples (Fig. S2). The total sequences per sample were considered in the microbial community composition, however, for diversity analyses, sequences were first rarefied to the minimum sequencing depth of 46 807 without significant loss to ASVs.

Community compositions at different temperature regimes

Four microcosms were sacrificed at the end of the treatment to investigate the community composition of replicates from treatments 1 (45°C), 2 (45 → 12°C), and 3 (45 → 25°C) at time points $t = 10$ d, 126 d and 82 d respectively (Fig. 1, Table S2), represented at the order

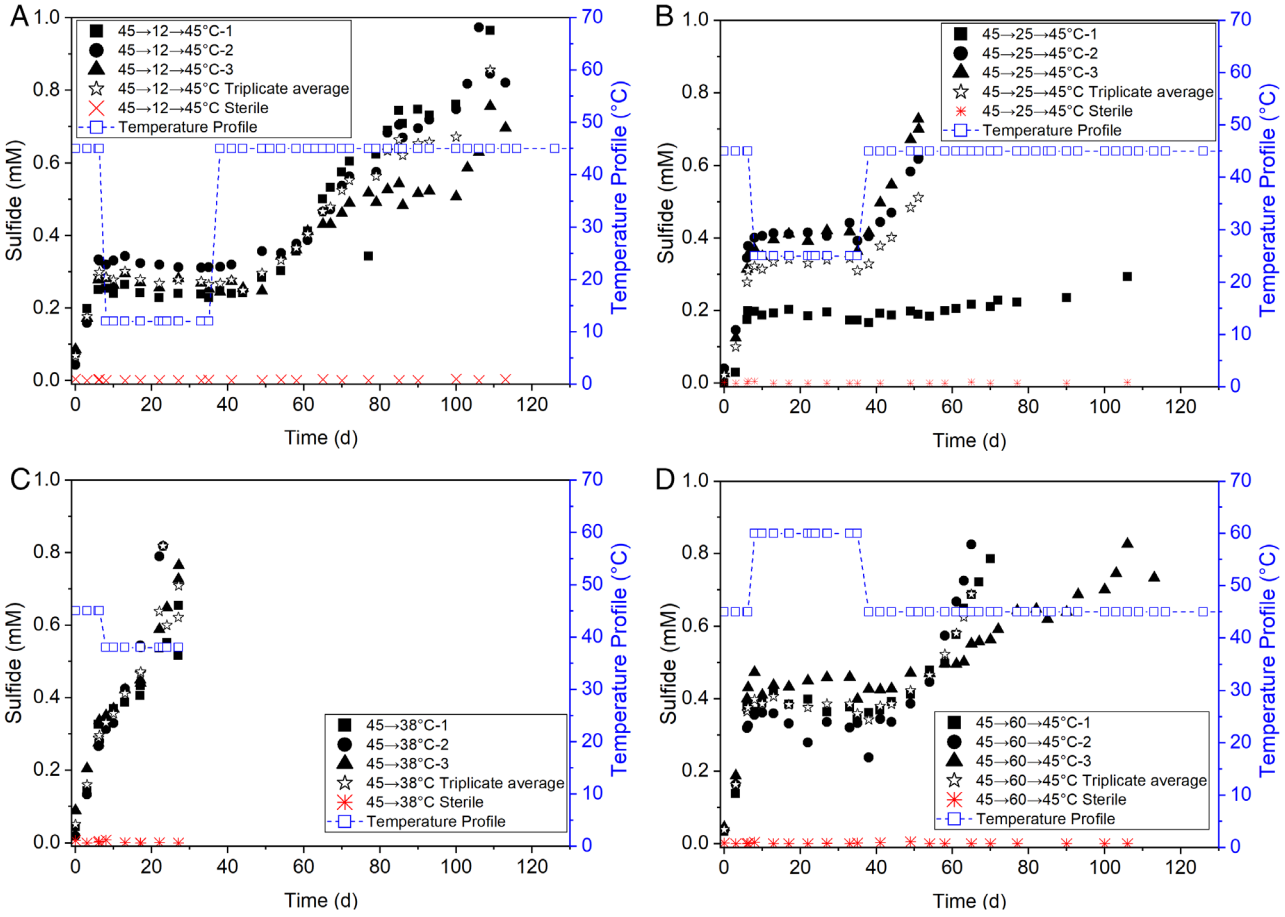


Fig. 3. Time courses on the sulfide production from microcosms in treatments 5 (45 → 12 → 45°C) (A), 6 (45 → 25 → 45°C) (B), 7 (45 → 38°C) (C) and 8 (45 → 60 → 45°C) (D). In treatment 7, the experiment was completed during incubation at 38°C and was not returned to 45°C.

and genus levels (Fig. 4A and B). The communities from treatment 4 (45 → 60°C) could not be sequenced due to insufficient DNA extracted from the biomass. In the community incubated continuously at 45°C, the dominant phylotypes belonged to the orders *Spirochaetales* (∼ 56%), *Clostridiales* (∼ 30%) and *Bacteroidiales* (9%) (Fig. 4A). At the genus level, members comprised mainly of uncultured members of the *Spirochaetaceae* (∼56%) and *Desulfotomaculum* (∼ 30%) (Fig. 4B). In replicate 4.07 of treatment 2 (45 → 12°C) sacrificed on day 126, and replicate 4.09 of treatment 3 (45 → 25°C) sacrificed on day 82, the orders *Spirochaetales* and *Clostridiales* were highly abundant as well (Fig. 4A). In treatment 2 (45 → 12°C), the dominant genera were affiliated with uncultured *Spirochaetaceae* (53%) and *Desulfotomaculum* (∼ 19%). *Candidatus* Desulforudis (∼19%), an uncultured bacterium of *Anaerolineae* (∼4%) and *Pelolinea* (∼4%) were more abundant in treatment 2, whereas these genera only ranged between 0.9% and 1.5% in treatment 1 (Fig. 4B). In contrast, several abundant phylotypes present in replicate 4.09 of treatment 3 (45 → 25°C) consisted of *Desulfotomaculum* (51%), uncultured *Spirochaetaceae* (21%) and *Symbiobacterium* (17%).

Eight replicates were sacrificed to investigate the community composition at the end of the experiment after the entire cycle of heat exchange (Figs. 5 and S3). In general, all replicates showed a high abundance of *Spirochaetales* (32%–61%) and *Clostridiales* (21%–56%) (Fig. S3). Other phylotypes which were present, albeit in varying abundances, included *Clostridia incertae sedis* (0.7% to ∼18%), *Bacillales* (0.5% to ∼4%) and *Bacteroidales* (0.01% to ∼8%) (Fig. S3). At the genus level, highly abundant phylotypes across the eight replicates were affiliated with *Desulfotomaculum* (∼15% to 56%) and *Candidatus* Desulforudis (∼0.8% to 18%) besides uncultured relatives of the *Spirochaetales* (Fig. 5). Several other phylotypes that were also present with varying abundances included a *Rikenellaceae* member (0%–8%), *Ruminococcaceae* (0%–9%) and *Ureibacillus* (∼1%–4%).

Three replicates were set up for each of treatments S1 (45 → 25 → 45°C) and S2 (45 → 60 → 45°C) to

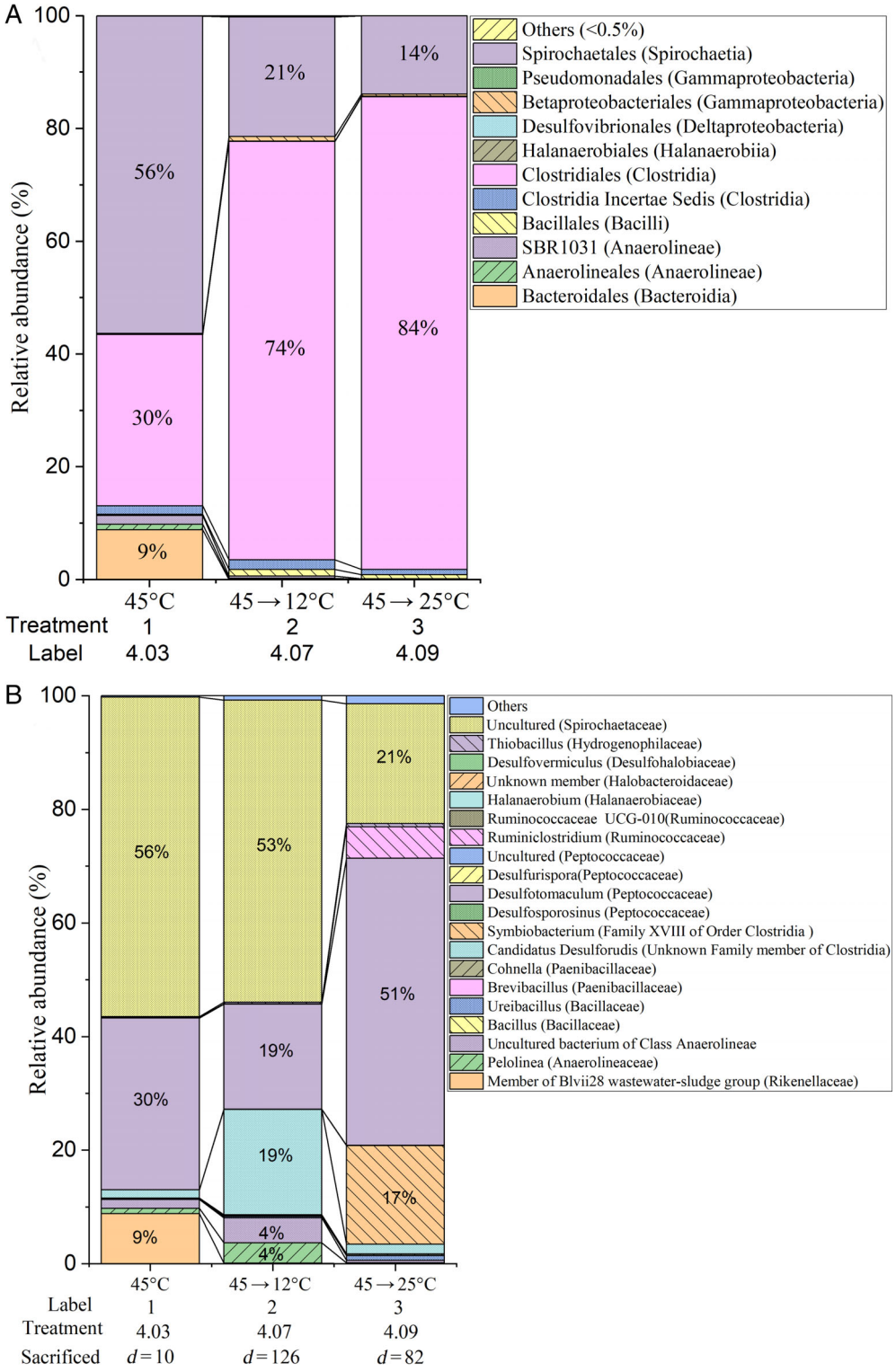


Fig. 4. Microbial community compositions for three treatments: 1 (45°C), 2 (45 → 12°C) and 3 (45 → 25°C), (A) represented at the order level (class level in parentheses), and (B) represented at the genera level (corresponding family level in parentheses). Community composition of treatment 4 (45 → 60°C) could not be obtained due to insufficient biomass.

investigate the community at each stage of the cycle with one replicate sacrificed each for pre-, post-temperature change and finally at the end of the cycle (Table S2). The replicates were monitored for acetate mineralization and sulfide production (Table 3) and behaved similarly as the replicates shown in Figs 3 and 4. Microbial community

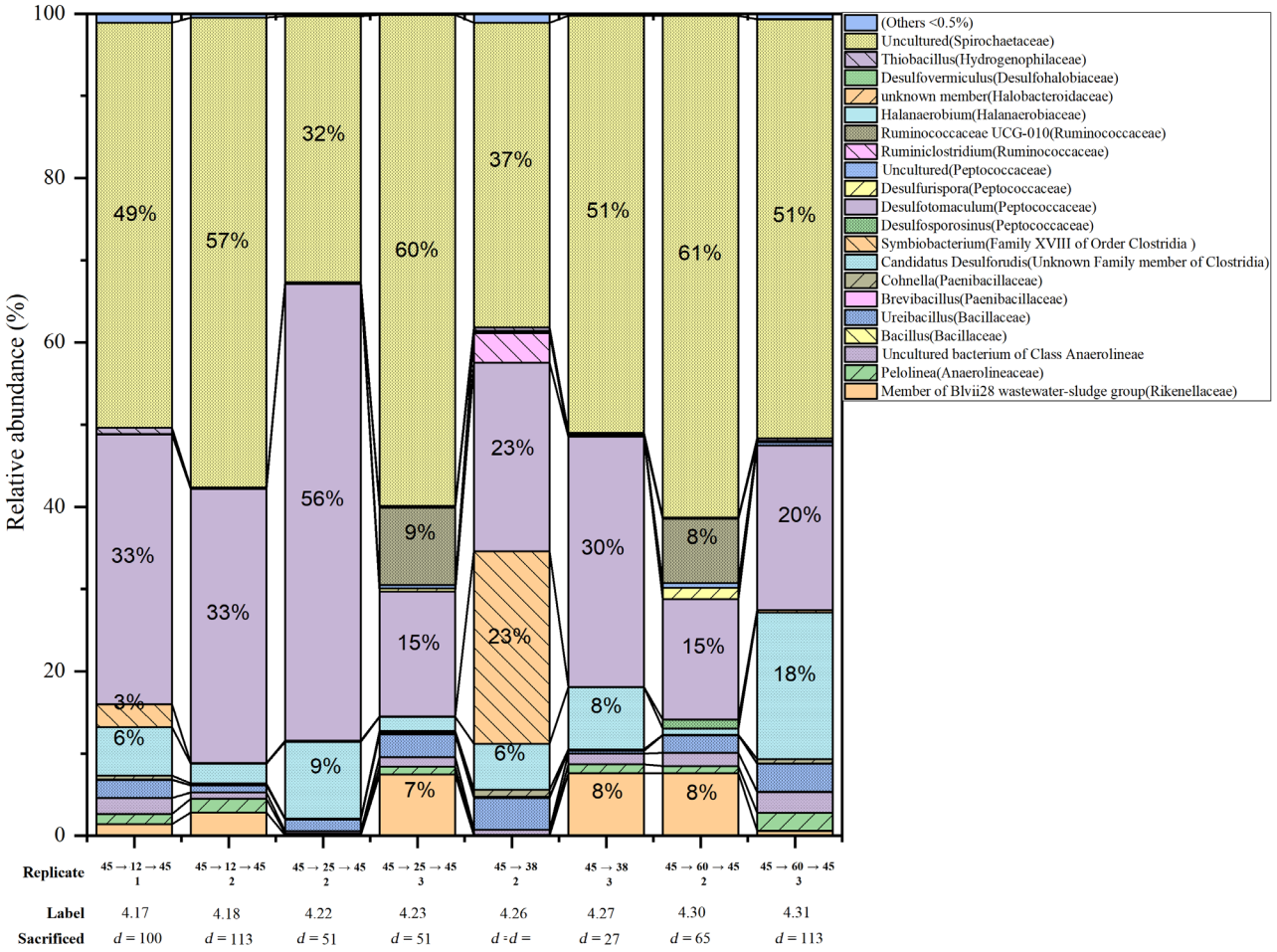


Fig. 5. Microbial community compositions of treatments: 5 (45 → 12 → 45°C), 6 (45 → 25 → 45°C), 7 (45 → 38°C) and 8 (45 → 60 → 45°C), represented at the genera level and the family level in parentheses. 'Replicate' and 'Label' information correspond to the replicate assignment described in Table S2. Two replicates were sacrificed from each treatment: 45 → 12 → 45°C (labels 4.17 and 4.18), 45 → 25 → 45°C (labels 4.22 and 4.23), 45 → 38°C (labels 4.26 and 4.27) and 45 → 60 → 45°C (labels 4.30 and 4.31). For consistency, replicates were numerically assigned (e.g., 45 → 12 → 45°C 1 or 45 → 12 → 45°C 2) corresponding to replicate assignments (Table S2). Two replicates, 4.26 and 4.27 were sacrificed at 38°C as the experiment was completed.

compositions of treatments S1 (45 → 25 → 45°C) and S2 (45 → 60 → 45°C) at genus level are represented in Fig. 6 (for compositions at the order level see Fig. S4). For treatment S1, phylotypes affiliated with uncultured members of the *Spirochaetaceae* (22%–35%) and *Desulfotomaculum* (22%–39%) were the most abundant genera across the three replicates subjected to the 45 → 25 → 45°C cycle. Several phylotypes such as *Pelolinea* (4%) and an uncultured member of the *Anaerolineae* (8%), were initially present at 45°C before temperature change at day 6; however, this diminished to 0.3%–1.1% at the end of the 45 → 25 → 45°C cycle, although *Pelolinea* abundance initially increased to ∼15% in the replicate 4.34 sacrificed after incubation at 25°C (Fig. 6). Meanwhile, *Candidatus* Desulforudis, which had a high relative abundance (∼18%) in replicate 4.35 (pre temperature change), saw a lower abundance (∼2%) in replicate 4.34 sacrificed at the end of the 25°C, which rebounded to ∼6% in replicate 4.33 sacrificed at the end of the 45 → 25 → 45°C cycle. Phylotypes such as *Brevibacillus* and *Bacillus* were initially present in the sacrificed replicate 4.35 ($d = 6$) but were subsequently absent in the replicates 4.34 ($t = 35$ d) and 4.33 ($t = 120$ d), while *Ureibacillus* abundance fluctuated slightly within the three replicates (Fig. 6).

In treatment S2 (45 → 60 → 45°C), phylotypes affiliated with the uncultured member of the *Spirochaetaceae* (38%–59%) and *Desulfotomaculum* (22%–44%) were the most abundant genera across the three replicates. Phylotypes affiliated to the *Rikenellaceae* (∼12%) and *Candidatus* Desulforudis (∼3%) were initially present at 45°C before temperature change at day 6; however, both taxa diminished to ∼1.0% at the end of the 45 → 60 → 45°C cycle. *Candidatus* Desulforudis abundance was however slightly higher (∼5%) in the replicate 4.37 sacrificed post-incubation at 60°C (Fig. 6).

Table 3. Overview on results of six replicates of acetate mineralization, sulfide production and rates for treatments S1 and S2.

Objective		Heat exchange					
	Treatment	S1			S2		
	Batch	45 → 25 → 45°C	45 → 25 → 45°C	45 → 25 → 45°C	45 → 60 → 45°C	45 → 60 → 45°C	45 → 60 → 45°C
	Label	4.35	4.34	4.33	4.38	4.37	4.36
Stage	Stage sacrificed	Pre	Post	End	Pre	Post	End
Pre	Duration (d)	6.25	6.25	6.25	6.25	6.25	6.25
	Acetate mineralized (%)	30.4	13.5	6.5	26.4	16.8	20.9
	Acetate mineralized (μM)	304	135.5	65	264.3	167.5	208.7
	Rate (μM acetate d^{-1})	48.6	21.7	10.4	42.3	26.8	33.4
	Duration (d)	6.25	6.25	6.25	6.25	6.25	6.25
	Sulfide produced (μM)	303	134.4	139.1	274.7	167.2	194
	Rate (μM acetate d^{-1})	48.5	21.5	22.2	44	26.7	31.0
Post	Duration (d)	Sacrificed	28.75	28.75	Sacrificed	28.75	28.75
	Acetate mineralized (%)	-	3.5	5.1	-	1.23[a.]	0.9[a.]
	Acetate mineralized (μM)	-	35	50.6	-	12.4[a.]	9[a.]
	Rate (μM acetate d^{-1})	-	1.2	1.8	-	-	-
	Duration (d)	-	28.75	28.75	-	28.75	28.75
	Sulfide produced (μM)	-	-	-	-	-	-
	Rate (μM sulfide d^{-1})	-	-	-	-	-	-
End	Duration (d)	Sacrificed	Sacrificed	85	Sacrificed	Sacrificed	47
	Acetate mineralized (%)	-	-	60.7	-	-	85.5
	Acetate mineralized (μM)	-	-	607.2	-	-	855.3
	Rate (μM acetate d^{-1})	-	-	7.1	-	-	18.2
	Duration (d)	-	-	81	-	-	47
	Sulfide produced (μM)	-	-	503.2	-	-	569.2
	Rate (μM sulfide d^{-1})	-	-	6.2	-	-	12.1

[a.] Acetate mineralization due to residual activity after shifting to 60°C.

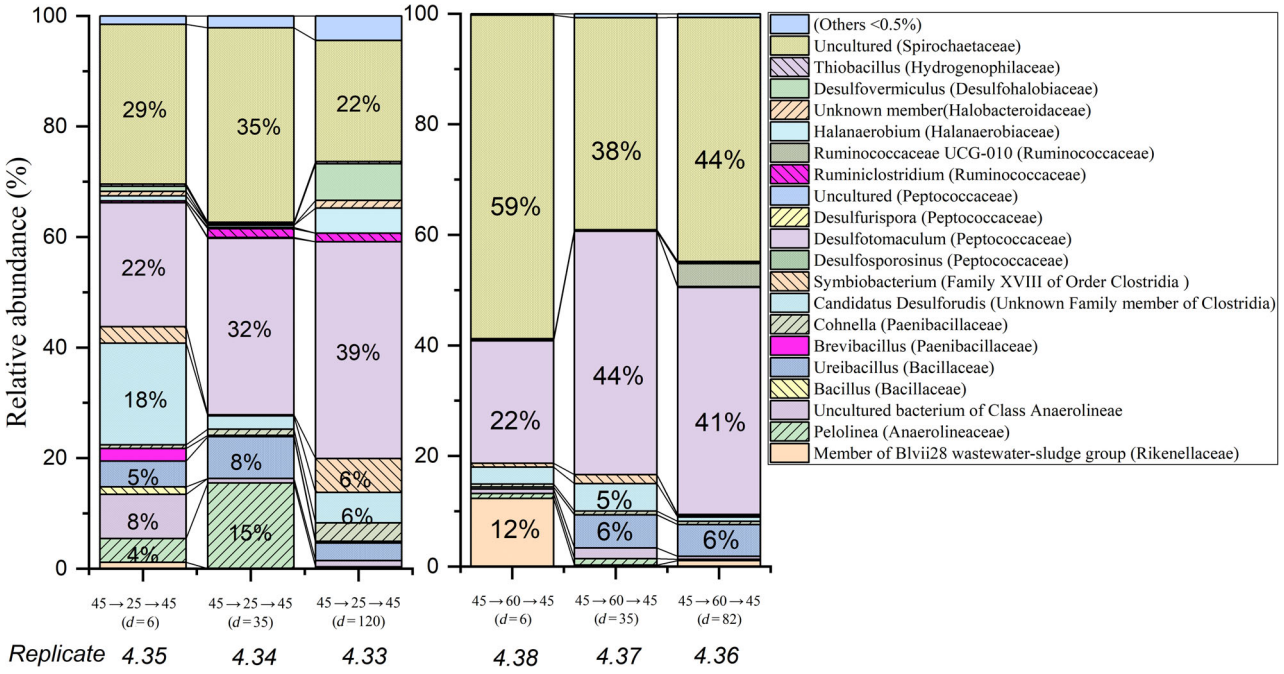


Fig. 6. Microbial community of the replicates sacrificed at various stages of the heat exchange, that is 'Pre' ($d = 6$), 'Post' ($d = 35$) and 'End' of the experiment ($d = 120$ and $d = 82$). Sequences are represented at the genus level, with the family level represented in parenthesis.

Microbial diversity indices of the differently treated communities are provided in Figures S6–S8.

Discussion

This study highlights the sulfate reduction potential of aquifer microbial communities at the boundary between mesophilic and thermophilic conditions. Acetate is a commonly reported intermediate during degradation of organic substrates (Thauer *et al*., 1989), for example in sulfidogenic benzene mineralization (Rakoczy *et al*., 2011; Starke *et al*., 2016), and was therefore used as a model carbon source for studying heterotrophic microbial activity in an aquifer microbial community originally adapted to anaerobic hydrocarbon degradation (Bin Hudari *et al*., 2020). Our data indicate that at 45°C (and also at 38°C, 25°C and 12°C), the bacterial community mineralized acetate with sulfate as the sole electron acceptor due to the ratio of produced sulfide to oxidized acetate of around 1 (Table 1) according to the following equation:

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$$

The sulfide production rate at 45°C (51.8 $\pm$ 3.8 μM d^{-1}) was approximately ten times higher compared to a previous study at the same temperature (Bin Hudari *et al*., 2020), illustrating that the enriched consortium was well adapted to 45°C. While sulfate reduction (SR) rates are often reported for typical mesophilic (e.g. 20–38°C) or thermophilic (e.g. 50–70°C) conditions (Isaksen *et al*., 1994; Elsgaard *et al*., 1994a,b; Kallmeyer and Boetius, 2004; Jesußek *et al*., 2012; Frank *et al*., 2015), rates for temperatures in between the meso-thermophilic range (~45°C) are scarce. The determined value for sulfide production d^{-1} in our study is in the range reported for SR rates between 42°C and 50°C ranging between ~25 and 316 μM sulfate d^{-1} (Frank *et al*., 2015; Robador *et al*., 2016). Alongside sulfide and labelled carbon dioxide production, the rate of pre- and post-temperature change reaffirms the impact periodic temperature fluctuations have on biotransformation. Cultures continuously incubated at 45°C were dominated by phylotypes distantly affiliated to the genus *Desulfotomaculum* and an uncultured member of the family *Spirochaetaceae*. *Desulfotomaculum* are sulfate reducers, which have been reported in various settings (Klemps *et al*., 1985; Bak and Pfennig, 1991; Aullo *et al*., 2013; Bonte *et al*., 2013; O'Sullivan *et al*., 2015; Tian *et al*., 2017). They are endospore formers (Widdel and Bak, 2006; Aullo *et al*., 2013; O'Sullivan *et al*., 2015; Grigoryan *et al*., 2018) and have been described at various temperatures including psychrophilic and thermophilic conditions (Klemps *et al*., 1985; Widdel and Bak, 2006; Aullo *et al*., 2013; O'Sullivan *et al*., 2015), thus, their higher

abundance at 45°C is not a surprise, and they are believed to oxidize acetate in our 45°C community, since several species such as *D. acetoxidans* and *D. kuznetsovii* are capable of utilizing acetate under sulfate-reducing conditions (Widdel and Pfennig, 1977; Thauer *et al*., 1989; O'Sullivan *et al*., 2015). However, the phylotype enriched in our 45°C culture was not clearly affiliated to known acetate mineralizing species of *Desulfotomaculum*, indicating that novel acetate-mineralizing *Desulfotomaculum* phylotypes could be responsible for the acetate-driven sulfate reduction at 45°C. A BLAST analysis of the 16S rRNA gene sequences (Table S4) revealed that the phylotype was affiliated to an uncultured *Sporotomaculum* sp. (99.8%) and to *Desulfallas thermosapovorans* DSM 6562 (98.5%) as closest relatives (Table S4). *Sporotomaculum* have been reported in setups with acetate as an electron donor under sulfate reducing conditions, but species reported from this genus are not known to be sulfate reducers (Brauman *et al*., 1998; Qiu *et al*., 2003; Purkamo *et al*., 2017; Hessler *et al*., 2020). Unlike *Sporotomaculum*, strains of *D. thermosapovorans* are sulfate reducers and with an optimal growth temperature of 50°C; however, they have not been described yet as acetate oxidizers (Fardeau *et al*., 1995). Meanwhile, the consistently high abundance of *Spirochaetaceae* across the 18 replicates (21%–61%) demonstrates they grew under the selected conditions. Although *Spirochaetaceae* have often been observed in anoxic habitats including sulfate-reducing conditions (Magot *et al*., 1997; Muller *et al*., 2009; Baldwin *et al*., 2016; Saad *et al*., 2017; Zouch *et al*., 2017; Dong *et al*., 2018; Bratkova *et al*., 2019; Bin Hudari *et al*., 2020; Ozuolmez *et al*., 2020; Yi *et al*., 2020), the capability for dissimilatory sulfate reduction has not been demonstrated in this family yet. In a naphthalene-degrading sulfate-reducing consortium, it was shown that a phylotype belonging to the *Spirochaetales* was recycling necromass by fermenting carbohydrates and proteins released from dead cells of the sulfate reducer and thereby producing hydrogen usable by the sulfate reducers (Koelschbach *et al*., 2017; Saad *et al*., 2017; Dong *et al*., 2018), and we assume therefore that necromass cycling was the main lifestyle of *Spirochaetaceae* in our enriched communities. Meanwhile, based on this study, there does not seem to be other predominant putative necromass recyclers capable of growth at 45°C, which implies that this phylotype is well suited for the mesophilic-thermophilic boundary (45°C) at anoxic conditions.

The key organisms in the 45°C consortium seem to be adapted to temperatures lying in the upper mesophilic to lower thermophilic zone. Some acetate mineralizers, *D. acetoxidans and D. thermoacetoxidans*, have an optimum growth temperature of 34–36°C (range: 20–40°C) and 55–60°C (range: 45–65°C) respectively (Min and Zinder, 1990; Stackebrandt *et al*., 1997; Widdel and Bak, 2006). Other sulfate reducers adapted to around 45°C that have not yet been shown to oxidize acetate are *D. geothermicum and D. sapomendans* with optimum temperatures of 54°C (range: 37–56°C) and 38°C (range: 20–43°C) respectively (Cord-Ruwisch and Garcia, 1985; Daumas *et al*., 1988; Widdel and Bak, 2006). Several *Anaerolineae* species were also reported to grow within the 25–60°C range, for example *Levilinea saccharolytica* KIBI-1 (optimum: 37°C, range: 25–50°C) and *Anaerolinea thermolimosa* IMO-1T (optimum: 50°C, range: 42–55°C) (Imachi *et al*., 2014). However, it is important to note that *Anaerolineae* were not particularly dominant in this study as compared to our earlier study where phylotypes belonging to this group were found to be dominant at 45°C under sulfate-reducing conditions (Bin Hudari *et al*., 2020), indicating that endospore-forming sulfate reducers and *Spirochaetaceae* are outcompeting *Anaerolineae* phylotypes after prolonged batch incubation at 45°C using acetate and sulfate as main carbon substrate and electron acceptor.

When exposed to a new temperature, a sustained mineralization of dissolved organic carbons (including pollutants) might be possible by either a succession of the microbial community resulting in the bloom of novel, temperature-adapted species occupying the same ecological niche, or by a temperature-specific intracellular adaptation of key species (Ranneklev and Baath, 2001; Delille *et al*., 2004). The latter is rather unlikely for broader temperature changes as prokaryotes are usually active in a temperature window of around Δ25°C (Madigan *et al*., 2017; see also discussion below). Hence, the observed resumption of activity at 12°C and 25°C can be best explained by a resilience of the original community to prolonged incubation at 45°C. However, the sequencing data did not clearly indicate a putative acetate oxidizer exclusively becoming enriched at 12°C or 25°C. Notably, the Desulfotomaculum phylotype, which is one of the putative key acetate degraders at 45°C and may be related to *Desulfallas thermosapovorans* (Table S4), was still abundant after mineralization of acetate at 12°C and 25°C (Fig. 4B); the relative abundance of this phylotype in treatment 3 (∼51%; 45 → 25°C) was also higher than in replicates of treatments 2 (∼19%; 45 → 12°C) and 1 (∼30%) incubated continuously at 45°C. One particular observation with respect to the community at the end of treatment 2 (45 → 12°C) was the higher abundance (∼19%) – compared to the setup incubated solely at 45°C – of a phylotype distantly related to *Candidatus* Desulforudis (Fig. 4). Only one species has been affiliated to *Candidatus* Desulforudis thus far, *Candidatus* Desulforudis audaxviator (∼93.0% identity, Table S4), which is

endospore forming and sulfate reducing (Jungbluth *et al*., 2017). *Candidatus* Desulforudis audaxviator was reported to grow between 45°C and 60°C with an optimal growth temperature of 55°C; however, it cannot grow with acetate (Karnachuk *et al*., 2019). In view of the relatively low percentage of identity, the enriched phylotype might be a new species of the genus *Candidatus* Desulforudis. This phylotype was also enriched at the end of incubation in the approaches 45-60-45, 45-38 and 45-25-45 (Fig. 5), pointing to an active role at 38–45°C rather than 12°C and 25°C. It is worth noting that since both phylotypes affiliated to *Desulfallas* and *Candidatus* Desulforudis were present in most of the treatments (Table S4), albeit at different relative abundances, we cannot exclude *per se* that both phylotypes were also active at 12°C and 25°C. Thus, the amplicon sequencing approach was not sensitive enough to differentiate between putative temperature-adapted subtypes. Other than that, the endospore survival strategy of both *Desulfotomaculum* and *Candidatus* Desulforudis could also have contributed to these phylotypes becoming more dominant at 45°C and staying dominant (at 45°C) even after the subsequent temperature changes in treatments 5 (45 $\rightarrow$ 12 $\rightarrow$ 45°C), 6 (45 $\rightarrow$ 25 $\rightarrow$ 45°C) and 8 (45 $\rightarrow$ 60 $\rightarrow$ 45°C). Notably, a phlyotype affiliated to the genus *Symbiobacterium* was abundant in two communities after longer incubation at 25°C (17%; treatment 3, Fig. 4) and 38°C (23%; treatment 7, Fig. 5), indicating an active role of this organisms for acetate oxidation at mesophilic conditions. *Symbiobacterium* is not known for sulfate reduction but has been identified to oxidize acetate syntrophically (Liu and Conrad, 2010).

In a previous study done by our group using an inoculum incubated for several years at room temperature ($\sim$ 20°C), completely different communities as well as sulfate reducers were identified (Bin Hudari *et al*., 2020). For example, several putative sulfate-reducing phylotypes related to *Desulfoprunum*, *Desulfovibrio* and *Desulfurivibrio* were present and more abundant in the 12°C and 25°C incubations while the *Desulfatiglans* and *Desufurivibrio* were present but in consistently lower abundances at 38°C (Bin Hudari *et al*., 2020). Sulfate reducers of the endospore-forming order Clostridia (e.g., *Desulfotomaculum*) were present in these setups but in markedly lower abundances (Bin Hudari *et al*., 2020). The considerable differences in the communities particularly the phylogenies of putative sulfate reducers in relation to this study further highlight the role of temperature in the succession of the native community.

Nevertheless, 45°C was the upper limit for acetate mineralization and sulfide production in our investigated community and within the experimental set up, and therefore thermophilic acetate degraders nor thermophilic sulfate reducers were probably not present, or present in such low numbers that the incubation time of this study was not long enough to detect their activity. However, active anaerobic thermophiles have been reported in aquifer sediments at 60°C (Bonte *et al*., 2013; Karnachuk *et al*., 2019), as well as aerobic thermophiles which seem to be more abundant in subsurface microbial communities (Metze *et al.*, 2021; Bin Hudari *et al*., 2020). Therefore, the question regarding the ubiquity of especially anaerobic thermophiles in shallow aquifer sediments needs to be explored in future (long term) studies.

Implications and outlook

To our knowledge, very few ATES-related studies have reported adaptation or recovery following periodic incubation at higher (heat storage) or lower temperatures (cold storage). From the ATES standpoint, 45°C is above that of the typical ATES threshold ($\sim$25°C) in Europe. However, this is considered to be a relevant temperature in HT-ATES systems, either in the operational space (the water that is usually extracted within the inner space of the system) in case of added heat up to 50°C, or in the outer, affected space in case of higher temperatures (above 60°C) in the operational space (Lerm *et al*., 2013; Kallesøe and Vangkilde-Pedersen, 2019). Our data indicate that natural aquifer communities harbor organisms well adapted to temperatures of the overlapping mesophilic-thermophilic boundary, sustaining the carbon cycle and being resilient to temperature fluctuations; overall, microbial activity could be sustained or resumed under the different temperature settings. This key finding indicates that cold storage (injection of cooler water during winter) or heat storage (injection of warmer water) ATES installations will change the structure of microbial communities but will not generally disturb microbial ecosystem services. However, the ultimate target remains to assess the feasibility of HT-ATES, using temperatures above, for example 60°C that would select for thermophilic or hyperthermophilic species. Our results suggest that above 60°C only a few (if any) thermophiles will be active in the operational space of a HT-ATES system. On the other hand, truly thermophilic communities may evolve at long-term operation of HT-ATES systems; especially at anoxic conditions, growth rates are usually low and a small inoculum of thermophilic initial community members may need several months or even years to considerably enrich. Additionally, inocula are probably site dependent, relying on a suitable microbial consortium to carry out biotransformations (Kallmeyer and Boetius, 2004). For example, communities isolated or well adapted in temperatures of up to 80°C have been described in in oil reservoirs of the deep subsurface, with the ability to degrade hydrocarbons (Head *et al*., 2014;

Piceno *et al*., 2014; Tian *et al*., 2017). However, it is unclear if these thermophilic organisms are present (albeit at very low numbers) in shallower aquifers and will only develop after a certain lag phase. Therefore, more studies utilizing different sediments are still required to investigate community resilience in the mesophilic-thermophilic boundary.

A higher threshold for LT-ATES to allow larger thermal energy storage while simultaneously remediating contaminants would be advantageous. Additionally, a more optimistic scenario of HT-ATES is where the society accepts that although the operational space of HT-ATES systems is being 'a microbial desert' and characterized by reduced or absent microbial activity, the (much bigger and less heated) affected space still contains active microbial communities which can perform ecosystem services. There is still some uncertainty how HT-ATES or higher threshold LT-ATES ($\sim$45°C, this study) could be maintained in the long term, where knowledge on community resilience and thermal cycling fatigue is still limited. The seasonal mode of ATES operations can cause fluctuations in the subsurface (Saito *et al*., 2016); therefore, the impacts of repeated heat exchange cycling at higher temperature thresholds need to be addressed. Furthermore, the effects of periodic heat exchange on nutrient availability and physicochemical characteristics of the contaminants should also be studied (Head *et al*., 2014). For example, an increase in temperature can result in lower organic carbon, total nitrogen or phosphorus (Jiao *et al*., 2016), while in others warming resulted in increases in carbon, total nitrogen and phosphorus (Geng *et al*., 2017; Gong *et al*., 2019). Understanding the overall impact of periodic heat exchange is an important step towards the goal of raising thermal storage thresholds. Multiple heat cycle experiments, combined with different heat exchange scenarios coupled with stable isotope probing, could also be applied to understand the long term effects of HT-ATES on ecosystem services and bioremediation.

Experimental procedures

Chemicals

Chemicals used were purchased from Sigma-Aldrich (USA), Merck (Germany) and Carl Roth (Germany) unless stated otherwise. Uniformly ^{13}C-labelled sodium acetate was purchased from Cambridge Isotope Laboratories (98% purity, 99 atom %, Massachusetts, USA).

Experimental setup

The inoculum used in this study belonged to an acetate-mineralizing sulfate-reducing enrichment culture (Zox4) consolidated from microbial communities of two microcosms (Zz-O1 and Zz-O2) comprising coarse sand obtained from the columns of an *on-site* system percolated with sulfidic benzene-contaminated groundwater, which were in operation for up to 10 years and 6 months at a mean temperature of 15°C as described elsewhere (Vogt *et al*., 2007; Taubert *et al*., 2012). The site, located north of the city of Zeitz in Central Germany, is contaminated for several decades by hydrocarbons and benzene due to former operations by an hydrogenation plant during world war II, and a benzene production plant between 1965 and 1990 (Schirmer *et al*., 2006). Zz-O1 and Zz-O2 were incubated since June 2010 at room temperature at sulfate-reducing conditions using benzene as substrate. Zz-O1 and Zz-O2 were believed to be slightly exposed to oxygen based on the presence of a lighter upper layer formed in the coarse sand with respect to the darker layer (Fig. S1). For preparing acetate-mineralizing enrichment cultures, the light, oxidized layers were removed from both Zz-O1 and Zz-O2 bottles and the dark, anoxic layers were distributed into six 0.5 l Schott bottles (Fig. S5) and filled with anoxic bicarbonate-buffered mineral salt medium (Bak and Pfennig, 1991) comprising 10 mM sulfate as electron acceptor and supplemented with 2 mM acetate as the electron donor (see Table S1 for composition). The new enrichment cultures were each placed at different incubation temperatures: 12°C, 25°C, 38°C, 45°C, 60°C and 80°C (labelled as Zox1, 2, 3, 4, 5, and 6 respectively), and monitored for sulfide production as an indicator for sulfate reduction activity (Fig. S5). Only cultures Zox1-4 (corresponding to 12°C, 25°C, 38°C, 45°C) produced sulfide (results not shown). For this study, the coarse sand of Zox4, which was enriched at 45°C for about 310 days, was used as an inoculum to set up 35 replicate bottles (Fig. S5); details of the replicate assignments can be found in Table S2. All replicates were prepared in 120 ml serum flasks made up of 5 g (treatments 1–8) or 15 g (treatments S1 and S2) coarse sand, and filled with 80 ml of modified carbonate-buffered mineral salt medium (Bak and Pfennig, 1991; see Table S1 for composition) amended with 10 mM sulfate and gas-tight sealed with Teflon-coated butyl rubber stoppers (Wheaton Industries, USA) and crimped aluminum caps. Treatment 1 served as 45°C reference (4.01 to 4.04 in Fig. S5) comprising three biotic replicates and one replicate (4.04) which was sterilized. In the 'adaptation' scenario, a total of nine biotic replicates were evenly distributed and firstly incubated at 45°C for 6 days before being transferred to 12°C for a further 120–127 days (treatment 2), to 25°C for up to 100 days (treatment 3) or to 60°C for 100 days (treatment 4) (4.05–4.15 in Fig. S5). Thus, an exception was made for treatment 2 (45 $\rightarrow$ 12°C) for additional incubation time, when mineralization commenced after $t = 70$ d allowing mineralization and sulfide

production to proceed (Fig. 1C and D). For the 'heat exchange' scenario, four replicates (three biotic and one sterilized) were each first incubated at 45°C for 6 days, followed by a periodic incubation for 28 days at 12°C (treatment 5), 25°C (treatment 6), 38°C (treatment 7) or 60°C (treatment 8), and finally returned to 45°C on $t = 35$ d for up to 78 days (4.17–4.32, Fig. S5). Replicates of treatments 1–8 were regularly sampled for sulfide and $\delta^{13}CO_2$ analyses; in some of the replicates, the microbial community was analysed at the end of the experiment (Fig. S5, Table S2A). Another six biotic replicates (4.33–4.38, Fig. S5) were set up for microbial community analysis at distinct time points of incubation using the entire bottle content ('phased sacrifice', Table S2B). Three replicates were assigned to each of treatments S1 (45 → 25 → 45°C) and S2 (45 → 60 → 45°C) to analyse community composition at different stages of the heat exchange (Pre, Post and End). For these treatments (S1 and S2), the first replicate was sacrificed at the end of the first incubation period (45°C, $t = 6$ d), the second at the end of the second temperature period (25°C or 60°C, $t = 35$ d, termed 'after') and the third at the end of the third temperature period (45°C, $t = 120$ d for replicate 4.33 and $t = 82$ d for replicate 4.36). Sulfide concentrations and $\delta^{13}CO_2$ values were analysed for each of the replicates.

Sterile controls were set up by autoclaving microcosms prepared as described above three times at 121°C for 20 min on three consecutive days. Prior to amendment of ^{13}C-labelled acetate (see below), microcosms were incubated at 45°C for three days to allow microcosms to thermally acclimatize. Uniformly ^{13}C-labelled sodium acetate from a sterile anoxic stock solution (100 mM) was added to each microcosm via a plastic syringe (B Braun, Germany) to a final concentration of 1 mM. Microcosms were incubated horizontally and statically in the dark at 12°C, 25°C, 38°C, 45°C or 60°C using different incubators (IN55, Memmert, Germany; Heraeus incubator, Thermoscience, USA). All used glassware was sterilized by autoclaving (121°C, 20 min) before usage. Microcosms were prepared in an anaerobic glove box (97% N_2 / 3% H_2 gas atmosphere, Coy Laboratory Products, USA) to avoid contamination by oxygen.

Chemical analyses

Analysis of $\delta^{13}CO_2$. Headspace samples (4 ml) were collected during the course of the experiment in N_2-flushed (2 min, 1 bar) glass vials (10 ml) via N_2-flushed syringes, which were crimped-sealed with Teflon-coated butyl rubber stoppers. Carbon isotope ratios of carbon dioxide for headspace CO_2 and CH_4 were measured by gas chromatography-combustion-isotopic ratio mass spectrometer (GC-IRMS) as described elsewhere (Bin Hudari *et al*., 2020).

The percent of mineralization (MIN %) of each replicate was calculated according to the following equation (Dorer *et al*., 2016):

$$\mathrm{MIN}\,(\%) = \frac{\left[\mathrm{HCO_3^-}\right] \times R_{\mathrm{VPDB}} \times (\delta_{\mathrm{end}} - \delta_{\mathrm{start}})}{(n-x) \times [\mathrm{acetate}] \times (1 + R_{\mathrm{VPDB}} \times (1 + \delta_{\mathrm{start}}))} \times 100 \quad (1)$$

Equation 1 includes isotope values (‰) of both the starting (δ_{start}) and end-point (δ_{end}), the concentrations of bicarbonate (HCO_3^-) and acetate as well as number of labelled carbon atoms of the target compound. The variables n and x represent the total and unlabelled carbon in the substrate respectively, where in the case of fully labelled acetate, $n - x = 2$. This value was used to compare the extent of acetate mineralization between treatments.

Analysis of sulfide. Sulfide was quantified in liquid samples using the modified methylene blue method originally described elsewhere (Cline, 1969; Muller *et al*., 2009). Up to 200 µl of samples were collected over the course of experiment, and samples were prepared as described in detail elsewhere (Bin Hudari *et al*., 2020). Calibration was carried out using a sodium sulfide nonahydrate stock solution (5 mM, Sigma-Aldrich, USA) made by dissolving it in 0.5 l of MilliQ spiked with one NaOH pellet (Carl-Roth, Germany) and flushed with nitrogen.

Microbial community analysis

Selected microcosms were sacrificed over time from each treatment temperature (Fig. S5, Tables S2A and S2B). Briefly, in treatments 1–8, one or two replicates were sacrificed at the end of the experiment. In treatments S1 and S2, one replicate was sacrificed for each of the pre, after and end stage of the heat exchange experiment (Fig. S5, Table S2B). Sessile cells were harvested from the coarse sand material via a series of sodium tetrapyrophosphate treatments using a modified method described elsewhere (Velji and Albright, 1984). First, 0.6 ml of sterile, cold 1% sodium tetrapyrophosphate (Sigma–Aldrich, USA) was added to each microcosm to a final concentration of approximately 0.01% (v/v) and sonicated for three pulses at 10 s each in an ice-water sonicating bath (Bandelin, Germany). The liquid was then transferred into new 50 ml Falcon tubes. This step was repeated for a further three times on the coarse sand: two rounds of sonication with 5 ml of 1% sodium tetrapyrophosphate for three pulses of 20 s each followed by a final round of sonication with 5 ml of 1% sodium tetrapyrophosphate

for one min. After each sonication round, the liquid was consolidated into the Falcon tubes and kept cool in an ice bath. At the end of the sonication step, the Falcon tubes with the consolidated liquid portions were centrifuged at 11000 r.p.m. at 4°C (Type 5804R, Eppendorf, Germany). The supernatant was discarded and the pellet was stored at −38°C in the freezer until DNA extraction. DNA was extracted from thawed cell-sand debris pellets using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and eluted in 30 µl of elution buffer. Extracted DNA was quantified with the Qubit HS (High Sensitivity) Assay kit (Thermo Fisher Scientific, USA) on the Qubit 3.0 Fluorometer (Life Technologies, Malaysia).

Procedures for MiSeq sequencing were similar to a study described elsewhere (Bin Hudari *et al*., 2020) using the same Klindworth primer pair (S-D-Bact-0341-b-S-17/ S-D-Bact-0785-a-A-21).

Sequencing libraries were assembled as described elsewhere using the Illumina MiSeq Reagent Kit v3 (2 x 300 bp) according to manufacturer recommendations on 16S Metagenomic Sequencing Library Preparations (Illumina, 2013) and sequenced on an Illumina Miseq platform at the Department of Environmental Microbiology of the Helmholtz Centre for Environmental Research -UFZ. Analysis of the sequences is similarly to that described by Bin Hudari *et al*. (2020) using QIIME 2 v2019.1 (Bolyen *et al*., 2019) with a pipeline provided by Dr. Denny Popp (UFZ, Department of Environmental Microbiology, Systems Biology of Microbial Communities Working Group). This includes first removing the primer sequences and adapters from the demultiplexed sequences, before trimming and denoising to remove low quality reads and chimeras before being merged. Amplicon sequence variants (ASVs) were assigned to the bacterial DNA against the silva132 database (Quast *et al*., 2013; Yilmaz *et al*., 2014). For analysis of Bray–Curtis dissimilarity and diversity analyses, samples were first rarefied to the minimum sequencing read with minor losses to the total amplicon sequencing variants ASVs (Fig. S2). Sequences were deposited at the European Nucleotide Archive (ENA) under the primary accession number PRJEB46801.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Two bottles, Zz-O1 and Zz-O2 used to further setup the six enrichment cultures Zox1-6. Most of the upper layers were discarded before consolidation of the darker layers.

Fig. S2. Rarefaction analysis of the 18 replicates sequenced.

Fig. S3. Microbial community compositions of treatments: 5 (45° → 12 → 45°C), 6 (45 → 25 → 45°C), 7 (45 → 38 → °C) and 8 (45 → 60 → 45°C), represented at the order level, with the corresponding class level in parentheses. 'Replicate' and 'Label' information correspond to the replicate described in Table S2.

Fig. S4. Microbial community of the replicates sacrificed at various stages of the heat exchange, that is pre-heat exchange ($d = 6$), post ($d = 35$) and at the end of the experiment ($d = 120$ and $d = 82$). Sequences are represented at the order level, with the class level represented in parenthesis.

Fig. S5. Summary of the workflow for enrichment of a community able to mineralize acetate at 45°C (culture bottle Zox4, used as the inoculum in this study), and the setup of differently treated replicate cultures in subsequent heat exchange experiments. Sterile controls are indicated by skulls. There are no replicates for labels 4.08, 4.12, and 4.16 as no sterile replicates were set up for treatment 2, treatment 3, and treatment 4 respectively.

Fig. S6. Shannon diversity index for twelve replicates including 4.03 to 4.31 in descending order.

Fig. S7. Diversity indices for replicates including 4.38 to 4.36 (A) and 4.35 to 4.33 (B) in descending order according to the stage of sacrifice. Replicate 4.03 was included as reference.

Fig. S8. PCoA of the 18 replicates from treatments 1-8, S1, and S2. Points are represented according to replicate (colour) and cluster (shape). Cluster 1 represents replicates from treatment 1; Cluster 2: 4.07, 4.09, 4.11; Cluster 3: 4.17–4.31; Cluster 4: 4.33–4.35; Cluster 5: 4.36–4.38.

Table S1. Recipe of the modified mineral salt media used for this study.

Table S2. Additional information for individual replicates for each treatment. Experiment in treatment 7 was completed during incubation at 38°C.

Table S3. Additional information for acetate mineralization, sulfide production for each replicate in treatments 1–8.

Table S4. Next relatives of the genera *Spirochaetaceae* member, *Desulfotomaculum* and *Candidatus Desulforudis* member phylotypes in this study for the select replicates, based on BLAST identities of 16S rRNA gene sequences. Phylotypes are arranged based on percent BLAST identity in descending order.

Chapter 4.

Sulfidic toluene mineralization by aquifer microbial communities at different temperatures

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Sulfidic toluene mineralization by aquifer microbial communities at different temperatures

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Abstract

High-temperature aquifer thermal energy storage (HT-ATES) is a carbon-neutral technology in the heating and cooling sector particularly suitable for urban areas, where aquifers are often contaminated with hydrocarbons. How HT-ATES could influence the natural degradation of contaminants such as hydrocarbons has hardly been investigated. Here, we determined the effects of temperature and temperature shifts on the capability of aquifer microbial communities to mineralize the model hydrocarbon toluene at sulfate-reducing conditions. Distinct toluene-mineralizing, sulfate-reducing consortia were enriched from material of two hydrocarbon-contaminated field sites at 12°C, 20°C, 25°C, 38°C, and 45°. Lowest toluene mineralization rates were observed at 38°C, and highest rates were observed at 45°C. Consortia adapted to 12°C or 25°C were generally negatively impacted by temporary or permanent temperature shifts to temperatures ≥ 38°C. *Desulfosporosinus* phylotypes dominated enrichments at 12°C, indicating a major role for toluene mineralization at *in situ* temperatures. At 20°C–25°C, typical sulfate-reducing genera such as *Desulfoprunum*, *Desulfallas* or *Pelotomaculum* were abundant, indicating synergistic relationships of various toluene degraders belonging to different taxa. The communities grown at 45°C were dominated by putative thermophilic phylotypes affiliated to the phyla *Bacillota* or *Caldiserica*. Overall, our data indicate that 45°C is the upper limit for anaerobic toluene mineralization of the investigated communities.

Keywords: high-temperature aquifer thermal energy storage; toluene mineralization; dissimilatory sulfate reduction; microbial community composition; groundwater; stable isotopes

Introduction

Aquifer thermal energy storage (ATES) is a promising low carbon energy storage technology in the heating and cooling sector (Lee 2010). Currently, 99% of ATES are low-temperature (LT-ATES) installations with operational thresholds of ∼25°C (Fleuchaus et al. 2018, Fleuchaus et al. 2020). Volatile commodity prices have rekindled interest in high-temperature (HT-ATES) that provide higher heat storage capacity (>40°C) and couple as sinks for excess heat generating industries (Fleuchaus et al. 2020). Nevertheless, the environmental impacts, operational costs, and regulatory matters of HT-ATES operations remain unclear (Fleuchaus et al. 2018, Fleuchaus et al. 2020).

ATES are generally constructed in urban and industrial areas with high energy demand that capitalize on their storage and supply capacity. These areas are typically contaminated with anthropogenically derived organic pollutants such as petroleum aromatic hydrocarbons (e.g. benzene-toluene-ethylbenzene-xylene [BTEX]), introduced via leakage or improper disposal (Rooney-Varga et al. 1999, Rittmann et al. 2000, Da Silva and Alvarez 2004, Vogt et al. 2007). In the subsurface, oxygen is rapidly depleted and abiotic processes are limited, hence hydrocarbon degradation is mainly characterized by biotic, anoxic reactions such as dissimilatory microbial sulfate reduction which are slower than aerobic biodegradation processes (Rittmann et al. 2000, Miao et al. 2012, Meckenstock et al. 2015, Lueders 2017). Although anaerobic hydrocarbon degrading microbial consortia and their pathways are well studied, subsurface ecosystems are microbially diverse and heterogeneous with localized niches; hence the site parameters may determine its biotransformation potential (Mancini et al. 2002, McKelvie et al. 2005, Gieg et al. 2010, Winderl et al. 2010, Fuchs et al. 2011, Meckenstock et al. 2015, Lueders 2017, Tian et al. 2017, Garcia et al. 2018, Pannekens et al. 2019).

Besides physicochemical contaminants characteristics, temperature also affects microbial mediated processes, where growth and activity decline rapidly and cease outside optimal environments (Higashioka et al. 2011, Huang et al. 2011, Schipper et al. 2014, Hossain et al. 2017, Madigan et al. 2018, Koproch et al. 2019). Indeed, studies focusing on the effects of increased temperatures on the anaerobic degradation potential of hydrocarbon-impacted aquifer communities are limited; a few showed that the degradation rates increased at higher temperatures, but that too high temperatures (up to 40°C) had an inhibiting effect on degradation (Zeman et al. 2014, Kulkarni et al. 2017). Some studies described how temperature selects for specific communities in habitats with temperature gradients, for example, microbial sulfate reduction was observed in the Guaymas Basin between 3°C and 95°C and in laboratories between 10°C and 102°C, comprising different sulfate reducers with temperature optima of 34°C, 70°C, and 80°C–88°C while hydrocarbon degraders up to about 70°C were enriched (Karl et al. 1988, Elsgaard et al. 1994, Kallmeyer and Boetius 2004, Teske 2024). The current data suggest that the thermal limit for hydrocarbon-degrading microorganisms in hot

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hydrocarbon reservoirs is between 80°C and 90°C (Aitken et al. 2004, Head et al. 2014, Pannekens et al. 2019). However, with regard to HT-ATES coupled with bioremediation in shallow aquifers where native temperatures are usually far below 30°C, it is currently not known whether thermophilic hydrocarbon-degrading microorganisms are present, or in which numbers, and whether thermophilic hydrocarbon-degrading microorganisms will be active if suitable conditions are created during an HT-ATES operation (Baas Becking and Canfield 2015).

Therefore, in this study, we investigated whether groundwater communities can degrade hydrocarbons at sulfate-reducing conditions at temperatures far above those in their natural habitat. Sulfate is a common electron acceptor in marine and freshwater systems (Jørgensen 1982, Holmer and Storkholm 2001, Zak et al. 2021). Toluene was selected because it is a common aromatic contaminant and well degradable at oxic and anoxic conditions in the mesophilic temperature range (Alvarez et al. 1991, Alvarez and Vogel 1991, Chen et al. 1992, Edwards et al. 1992, Langwaldt and Puhakka 2000, Roychoudhury and McCormick 2006, Roychoudhury and Merrett 2006, Winderl et al. 2008, Meckenstock et al. 2015); moreover, its anaerobic degradation pathway is well understood (Lueders 2017). Degradation of toluene at sulfate-reducing conditions under thermophilic conditions (50–70°C) was reported once (Chen and Taylor 1997), performed by microbial consortia from hot oil reservoirs. Hence, no data are available on the anaerobic degradation potential of aquifer communities at thermophilic conditions from temperate zones where temperatures are permanently moderate. In our study, we selected five different temperatures based on the following rationales: 12°C (native aquifer conditions), 25°C (upper temperature limit for LT-ATES operations in many countries), 38°C (HT-ATES conditions, upper limit for mesophilic conditions), 45°C: (HT-ATES conditions, lower limit for thermophilic conditions), and 60°C (HT-ATES conditions, thermophilic conditions). During HT-ATES operations, the selected temperatures can be attained in the core zone (hottest area) or along a temperature gradient which develops in the surrounding zone (Lerm et al. 2013, Daniilidis et al. 2022). Aquifer microbial communities were obtained from package materials (gravel) derived from a former *in situ*-column experiment at a hydrocarbon-contaminated aquifer (Zt) and from sediments from a hydrocarbon-impacted aquifer (Wg). These were used to set up enrichment cultures at the selected temperatures at sulfate-reducing conditions with toluene as a sole source of carbon and energy. Key players of toluene mineralization at different temperatures were identified by 16S rRNA gene analysis. Active cultures were subjected to temperature changes in additional experiments aiming to simulate different ATES treatment regimes, testing the resilience of the temperature-adapted toluene-mineralizing consortia to temporary or permanent temperature fluctuations.

Materials and methods

Chemicals were purchased from Sigma-Aldrich (USA), Merck (Germany or Switzerland), and Carl Roth (Germany), unless stated otherwise. [^{13}C]-α-toluene was purchased from Sigma-Aldrich (99% purity, 99 atom % at α position, Missouri, USA).

Culture setup

Initially, seven different enrichment cultures were set-up at several temperatures using gravel from the Zeitz site (Zt) and aquifer sediments from the Weißandt-Gölzau site (Wg) (Fig. S1_Supplementary Data).

Set-up of toluene-amended enrichment cultures with Zeitz gravel

Gravel was collected from columns used in an *on-site* percolation installment with sulfidogenic benzene-contaminated groundwater operated for 10 years and 6 months at around 15°C as described elsewhere (Vogt et al. 2007, Taubert et al. 2012). The gravel was obtained in January 2017 when the column experiment was finished and incubated thereafter in the laboratory in 1-L Schott bottles at room temperature (20°C) with anoxic bicarbonate-buffered mineral salt media comprising 10-mM sulfate and 1-mM benzene in 5 mL of the inert carrier phase 2,2,4,4,6,8,8-heptamethylnonane (HMN) and maintained initially as a benzene-amended enrichment culture and constantly replenished with benzene and sulfate. In June 2019, in two bottles, the mineral salt media were discarded, the gravel was mixed in a beaker, 400-g gravel were redistributed each into six 500-mL Schott bottles, and amended each with 300-mL BUS medium (BUS = Benzene Utilizing Sulfate Reducer medium; Table S1_Supplementary Data), supplemented with HMN (5 mL) and 5 µL of toluene, and sealed gas-tight with butyl stoppers fixed with screw caps. The cultures were incubated at six different temperatures (12°C (Zt12), 20°C (Zt20), 25°C (Zt25), 38°C (Zt38), 45°C (Zt45), 60°C (Zt60)) using incubators from the companies Heraeus (Germany, model B6060; for 20°; 25°C and 38°C), Memmert (Germany, model IN30; for 45°C and 60°C) and Binder (Germany, cooling incubator; for 12°C) and maintained for 21 months. Sulfate-reducing activity was monitored by sulfide production. Of the six bottles started, activity could be determined only in Zt12, Zt20, and Zt25 (results not shown).

Set-up of toluene-amended enrichment cultures with Weißandt-Gölzau aquifer sediment

Sediments were obtained from a hydrocarbon-contaminated aquifer in Weißandt-Gölzau (51°40′N 12°4′E) in Saxony-Anhalt, Germany, a former site of a brown coal processing industry where sulfate reduction and methanogenesis were considered among the predominant electron accepting processes in the aquifer (Feisthauer et al. 2012). Sediments were collected during a drilling campaign in July 2012, placed in air-tight jars and stored at anoxic conditions at 4°C. In April 2019, enrichment cultures were set up, using sediments sampled from a well at a depth of 8.5–9.5 m. Enrichment cultures were prepared by mixing 3 kg of sediment and 100-mL BUS medium (Table S1_Supplementary Data) in a sterile beaker, subsequent distribution of the resulting slurry (~510 g each) in five 1-L bottles (Schott, Germany) topped up to around 1 L; each bottle was amended with 5-mL HMN and 5-µL toluene, closed gas-tight with butyl stoppers and fixed with screw caps. The cultures were incubated statically in the dark at five different temperatures, namely, 12°C (Wg12), 25°C (Wg25), 38°C (Wg38), 45°C (Wg45), and 60°C (Wg60), using the same incubators as for the Zt enrichments. Sulfate-reducing activity, determined via sulfide production, was observed in Wg12, WG25, WG38, and WG45 after prolonged incubation. These cultures were further maintained by the addition of toluene (~ 5 µL, neat) for at least 15 months before proceeding further (see below).

Preparation of microcosms used for controlled laboratory experiments

From the active sulfate-reducing enrichment cultures, microcosms for monitoring toluene mineralization were prepared as follows: 5 g of gravel or sediment of a culture was distributed each in a 120-ml serum bottle and around 80-mL BUS medium

containing 10-mM sulfate (Table S1_Supplementary Data) was added. The microcosms were sealed gas-tight using butyl septa and aluminum crimps. Prior to toluene addition, all microcosms were incubated at the respective initial temperatures of 12°C, 25°C, or 38°C for three days to condition them at the experimental temperatures before toluene addition. Next, each replicate was amended with 3 mL sterile autoclaved HMN via a plastic syringe (B Braun, Germany) followed by addition of [^{13}C]-α-toluene to a final concentration of 1 mM with a glass syringe (Hamilton, USA). Toluene mineralization and sulfate reduction were generally monitored by analyses of $\delta^{13}CO_2$ and sulfide (see below). Thirty-six replicates were each set up with the initial cultures Zt12 (Table S2_Supplementary Data) and Zt25 (Table S3_Supplementary Data), respectively, 45 replicates were set up with each initial cultures Wg12 (Table S4_Supplementary Data), Wg25 (Table S5_Supplementary Data), and Wg38 (Table S6_Supplementary Data). Three replicates were set up for Zt20°C (Zt20), and seven replicates (including one sterile replicate) were set up for Wg45 (Table S7_Supplementary Data). Active enrichment cultures were selected to compare their toluene mineralization and sulfide production rates, and microbial communities at their enrichment temperature. Additional experiments with regard to temperature 'adaptation' (cultures exposed permanently to a different temperature) and 'heat exchange' (cultures exposed temporarily to a different temperature) were set up using cultures Zt12, Zt25, Wg12, Wg25 and Wg38 to observe short- and long-term effects of temperature stress on temperature-adapted communities.

All microcosms were prepared in a glove box (5% N_2/5% H_2 gas atmosphere, Coy Laboratory Products, USA) under anoxic conditions using sterile equipment. All equipment was generally sterilized by autoclaving (121°C, 20 min).

Chemical analyses

$\delta^{13}CO_2$ analyses

Headspace samples were collected with N_2-flushed plastic syringes and stored in N_2-flushed glass vials (5 mL) crimped with Teflon-coated butyl rubber septa and aluminum crimps before analysis. Measurements of CO_2 and CH_4 carbon isotope ratios were conducted by gas chromatography-combustion-isotopic ratio mass spectrometry (GC-IRMS) consisting of a gas chromatograph (Agilent, USA) containing a PoraplotQ column (25 m × 0.32 mm ID, 1-µM film; Chrompack, Middleburg, USA) coupled to a mass spectrometer via a Conflow III interface (Germany). Carbon dioxide separation was conducted isothermally at 40°C with helium carrier gas at a flow rate of 2 mL min^{-1}. Post-separation products were transferred to a combustion furnace (GC/C-III; Thermo Fisher Scientific, Bremen, Germany) maintained at 980°C on a CuO/Ni/Pt catalyst that also tracks carbon isotope signatures of methane converted to carbon dioxide, although this was not detected in any of the samples. Samples with varying volumes (50–500 µL) were injected at 1:5 split ratios. Carbon isotope signature data are shown in delta notation (per mille, ‰) relative to the Pee Dee Belemnite standard.

The d^{13}C/^{12}C data in per mille (‰) were used to calculate the extent of mineralization (MIN %) of each replicate using the following equation (Dorer et al. 2016):

$$\text{MIN } (\%) = \frac{\left[HCO_3^-\right] \times R_{VPDB} \times (\delta_{end} - \delta_{start})}{(n-x) \times [toluene] \times (1 + R_{VPDB} \times (1 + \delta_{start}))} \times 100. \quad (1)$$

Equation 1 incorporates the δ values at the start and end of the experiment, estimated starting toluene concentrations based on the injection volume, and the variables n and x which represent the total and non-labelled carbon of the target compound, respectively. In this experiment, [^{13}C]-α-toluene is the target substrate, with only single labelled carbon, hence $n = 7$ and $x = 1$. A previous study has shown that the methyl moiety of [^{13}C]-α-toluene was better assimilated into the total lipid fatty acid (8–15%) compared to the fully labelled [$^{13}C_7$]-toluene (51–57%) (Bombach et al. 2010). Since the methyl moiety can end up as acetyl-CoA, precursors for fatty acids or carbon dioxide, [^{13}C]-α-toluene is suitable to obtain mineralization data (Heider 2007, Lueders 2017).

The general equation for complete toluene mineralization and coupled sulfate reduction (linked to stoichiometric sulfide production) is as follows (Beller et al. 1992):

$$C_7H_8 + 4.5SO_4^{2-} + 3H_2O \rightarrow 2.25H_2S + 2.25HS^- + 7HCO_3^- + 0.25H^+. \quad (2)$$

Toluene mineralization and sulfate reduction associated to cell growth can be described by (Beller et al. 1996)

$$C_7H_8 + 4.09SO_4^{2-} + 0.17NH_4^+ + 2.49H_2O \rightarrow 2.04H_2S + 2.04HS^- + 0.17C_5H_7O_2N\ (cells) + 6.16HCO_3^- + 0.2H^+. \quad (3)$$

Sulfide analysis

Sulfide was quantified similarly to our previous studies via the modified methylene blue method described elsewhere (Cline 1969, Müller et al. 2009, Bin Hudari et al. 2022). Samples (50–200 µL) were collected over the course of the experiment via sterile N_2-flushed syringes, added immediately to 1 mL of 3% zinc acetate dihydrate in glass test tubes followed by the consecutive addition of 4 mL bi-distilled water and 400 µL Cline reagent prepared as previously reported (Bin Hudari et al. 2022). After reagent addition, the mixture was mixed briefly, incubated in the dark for 20 min, mixed again and transferred to 2.5 mL cuvettes for absorbance measurement at 670 nm using a spectrophotometer (Novaspec III, Amersham Biosciences, Cambridge, United Kingdom) against a blank reference made of bi-distilled water.

Microbial community analysis

Microcosms were sacrificed for microbial community analyses either at the end of the incubation period or if $^{13}CO_2$ or sulfide production data for the replicate suggested that activities had ceased. Cells were harvested from solid materials (gravel or sediment) via a series of sodium tetrapyrophosphate modified treatment protocol as described elsewhere (Bin Hudari et al. 2022). Upon de-capping, ~0.6–0.8 mL of sterile cold 1% sodium tetrapyrophospate (Sigma-Aldrich, USA) was added to each microcosm to a final concentration of ~0.01% (v/v) and sonicated for three pulses of 10 s in an ice-water sonicating bath (Bandelin, Germany). The liquid fraction was poured into new pre-labelled 50 mL Falcon tubes. Five mL of 1% sodium tetrapyropshophate was added to the sediment and subjected to three pulses of 10 s of sonication in ice-water bath, and the liquid fraction was transferred to Falcon tubes. This step was repeated twice at 20-s pulses followed by a final pulse of 60 s. After each run, the liquid was transferred to Falcon tubes and kept cool in an ice bath. Falcon tubes were then centrifuged at 11 000 rpm at 4°C (Type 5804 R, Eppendorf, Germany). The supernatant was discarded and the pellet was stored at −38°C until DNA extraction. Extraction was carried out with the DNEasy Powersoil Kit (Qiagen, Hilden, Germany) mainly as per the manufacturer's instructions. Prior to

DNA extraction, the tubes were first allowed to thaw for at least 30 min at 4°C, and the pellets were re-suspended with Powerbead Tube Buffer in the Falcon tubes before transfer, if necessary, more than one tubes were used per replicate. Samples were combined during the column spinning process to collect the precipitated DNA and finally eluted to ~20–30 µL. Extracted DNA concentration was quantified with the Qubit HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, USA) on the Qubit 3.0 Fluorometer (Life Technologies, Malaysia). MiSeq sequencing procedures were similar to previous studies prescribed elsewhere (Bin Hudari et al. 2020, 2022) with the Klindworth primer pair (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) (Klindworth et al. 2013). Briefly, sequencing libraries were assembled with Illumina MiSeq Reagent Kit v3 (2 × 300 bp) as per manufacturer recommendations on 16S Metagenomic Sequencing Library Preparations (Illumina, 2013) and sequenced on the Illumina Miseq platform at the Department of Applied Microbial Ecology of the Helmholtz Centre for Environmental Research - UFZ. Sequences were analysed using QIIME 2 2025.4 (Bolyen et al. 2019) using a pipeline provided by Dr. Denny Popp. This pipeline includes removing primer sequences and adapters from the de-multiplexed sequences, trimming, and denoising to remove low-quality reads and chimeras, before merging. The silva138.2 database was used to assigned amplicon sequence variants (ASVs) to the bacterial DNA (Quast et al. 2013, Yilmaz et al. 2014). Prior to dissimilarity (α and ß) diversity analyses, samples were first rarefied to the number of reads of the sample with the least amount of sequences. Sequences were deposited at the European Nucleotide Archive (ENA) under the primary accession number PRJEB77513.

Statistical analysis

To analyse the differences in median rates for Zt and Wg inocula at various temperatures, the toluene mineralization and sulfide production rates in Zt ($n = 9$) and Wg ($n = 16$) groups were tested using the Wilcoxon paired sample test with Bonferroni–Hochberg correction. A P value < 0.05 was considered statistically significant. All statistical analyses were done with the statistical computing software R (https://www.r-project.org/).

Results

Toluene mineralization and sulfide production at different temperatures and temperature change scenarios

Considerable sulfide production was observed in seven enrichment cultures: Zt12, Zt20, Zt25, Wg12, Wg25, Wg38, and Wg45; no cultures could be established at 60°C with both used materials, and at 38°C and 45°C with Zeitz gravel within prolonged (>1 year) incubation (data not shown).

Toluene mineralization and sulfide production rates at constant temperature

[^{13}C]-α-toluene mineralization and sulfide production rates of the seven setups (Zt12 to Wg45) are consolidated in Fig. 1 (for the complete data set, see Tables S8-S9_Supplementary Data and Figs S2-S5_Supplementary Data).

Microcosms incubated between 12 to 25°C (Zt12, Zt20, Zt25, Wg12 and Wg25) showed roughly comparable rates for toluene mineralization (10.7 ± 2.9–16.2 ± 0.6 µM d^{-1}) and sulfide production (43.4 ± 1.5–73.6 ± 2.2 µM d^{-1}). At 38°C (Wg38), toluene mineralization and sulfide production rates were around 4–6 and 6–10 folds lower, compared to the setups incubated at 12°C and 25°C, respectively, indicating non-optimal condition. The highest toluene mineralization and sulfide production rates were observed at 45°C (Wg45) that were around 2–4 folds (40.3 ± 0.9 and 178.5 ± 4.2 µM d^{-1}) higher compared to the setups incubated at 12°C and 25°C, respectively. The pairwise Wilcoxon test indicated no significant differences ($P > 0.05$) in the median rates of both toluene mineralization and sulfide production between the Zt cultures at 12°C, 20°C, and 25°C. In contrast, significant differences ($P \leq 0.05$, '$*$') were observed in the median rates of toluene mineralization and sulfide production between the inoculum cultures at 12°C, 25°C, 38°C, and 45°C (Fig. 1).

Based on Equation (2), the expected ratio of sulfide produced to toluene mineralized is 4.5 in the case of complete mineralization. Of the seven ratio averages, five values were close to the expected ratio (4.0–4.8). Zt25 setups had the highest average ratio (6.0), whereas Wg38 had the lowest ratio of 2.7 (Table S9_Supplementary Data).

Effects of permanent temperature change on temperature-adapted cultures ('adaptation' scenario)

Active Zt12, Zt25, Wg12, WG25, and Wg38 cultures, firstly incubated at 12°C, 25°C, or 38°C for 28–34 days with [^{13}C]-α-toluene, were transferred permanently to higher or lower temperatures for more than 100 days to determine immediate and long-term effects of temperature changes on toluene mineralization and sulfide production rates of the temperature-adapted enrichment cultures.

Zt12 setups switched to 25°C developed similarly as those incubated at 12°C (Fig. 2). When Zt12 cultures were moved to 38°C, toluene mineralization and sulfide production initially stopped but resumed at lower rates around 100 d after shifting to 38°C (Figs 2A; S6B, S7B_Supplementary Data). Zt12 cultures lost toluene mineralization activity when placed at 45°C or 60°C (Fig. 2A). Cultures adapted to 25°C (Zt25) showed lower toluene mineralization and sulfide production rates when shifted to 12°C and 38°C (Fig. 2B). At 38°C, toluene mineralization and sulfide production later ceased following prolonged incubation (Figs S8B, S9B_Supplementary Data). When shifted to 45°C and 60°C, Zt25 cultures mineralized toluene no longer (Fig. 2B).

Rates of Wg12 setups adapted at 12°C increased when transferred to 25°C while exposures to temperatures higher than 25°C almost completely inhibited toluene mineralization; only a single replicate exposed to 38°C showed sustained but lower toluene mineralization rates (Figs 3A, S10B_Supplementary Data). In Wg25 setups transferred from 25°C to 12°C, rates of toluene mineralization and sulfide production significantly decreased (Fig. 3B). In Wg25 setups transferred to 38°C, cultures stopped mineralizing toluene within 17 days (Fig. 12B_Supplementary Data); when exposed from 25°C to 45°C or 60°C, toluene mineralization ceased rapidly (Fig. 3B). Toluene mineralization and sulfide production rates at 38°C (Wg38) were generally considerably lower compared to cultures enriched at 12°C, 25°C, or 45°C (Fig. 1). When the cultures were moved to 25°C, activity was sustained but rates decreased (Figs 14B, 15B, 16_Supplementary Data). The Wg38 setups lost toluene mineralization activity when exposed to 12°C, 45°C, or 60°C (Figs S14, S15ACD, 16_Supplementary Data).

Effects of transient temperature change on temperature-adapted cultures ('heat exchange scenario')

Cultures Zt12, Zt25, Wg12, Wg25, and Wg38 were incubated for 28–34 days at 12°C, 25°C, or 38°C, respectively, then temporarily transferred to higher or lower temperatures for 14 days, and re-shifted to the initial incubation temperature.

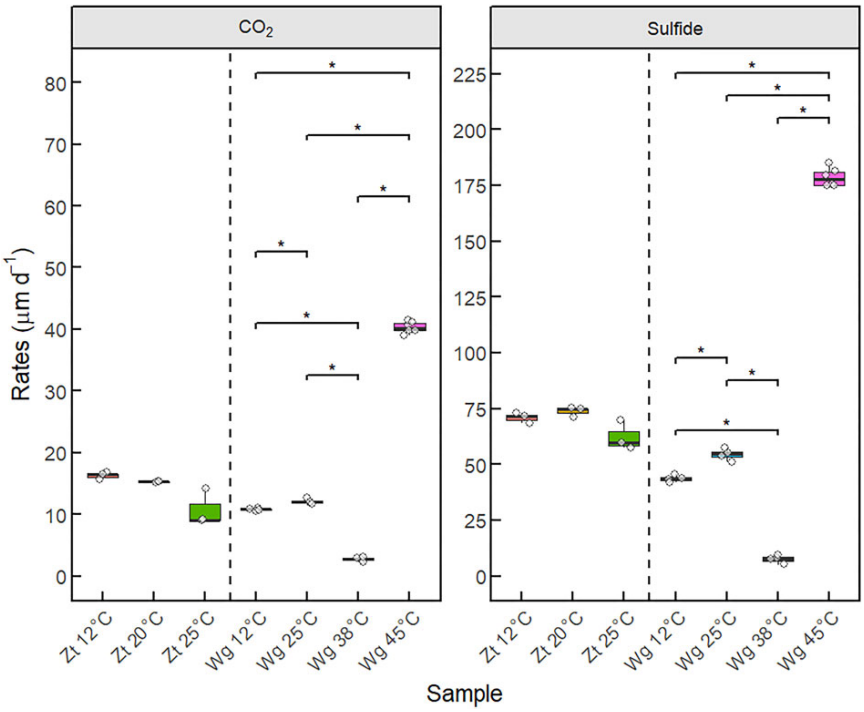


Figure 1. Boxplots of toluene mineralization and sulfide production rates, represented as average values ($n \geq 3$). A Wilcoxon paired test was performed between the inocula with the sediment groups Zt and Wg, respectively. * indicates statistical significance ($P \leq 0.05$).

Zt12 cultures incubated temporarily at 25°C preserved toluene mineralization and sulfide production rates (Fig. 2A). However, Zt12 cultures placed transiently at 38°C, 45°C, or 60°C lost toluene mineralization activity after temperature change (at 38°C, activity sustained for a couple of days); only cultures temporarily shifted to 38°C or 45°C began mineralizing toluene and producing sulfide 60–90 days after shifting back to 12°C (Figs 2A; S17, S18_Supplementary Data). In most Zt25 cultures transiently shifted to 12°C or 38°C, toluene mineralization and sulfide production rates decreased during the temperature shift phase (Fig. 2B) but activities regained when shifted back to 25°C (Figs 2B; S19AB, S20AB_Supplementary Data). Zt25 cultures transiently exposed to 45°C or 60°C lost toluene mineralization activity completely during the increased temperatures phase and afterwards, except for a replicate of the 25→45→25 approach that regained toluene mineralization and sulfide production activities more than 60 days after being returned to 25°C (Figs 2B; S19CD, S20CD_Supplementary Data).

Wg12 cultures mineralized toluene and produced sulfide at an accelerated rate when placed temporarily at 25°C (Fig. 3A). Higher temperatures (38°C, 45°C or 60°C) resulted in rapid or slightly delayed loss of toluene mineralization during exposure and, for a certain time, after resetting the temperature to 12°C (Figs 3A; S21BCD, S22BCD_Supplementary Data). Some of the 38°C and 45°C exposed replicates regained toluene mineralization activity more than 60 days after the temperature shift phase (Figs 21BCD, S22BCD_Supplementary Data). In Wg25 cultures, a temporary decrease in temperature to 12°C resulted in slightly lower toluene mineralization and sulfide production rates (Fig. 3B). In WG25 cultures temporary shifted to 38°C, toluene mineralization and sulfide production rates strongly decreased a couple of days after the shift to 38°C, and the rates remained at this low level for more than 100 days, until the end of incubation (Figs 3B; S23B, 24B_Supplementary Data). Wg25 cultures temporary shifted to 45°C or 60°C rapidly lost toluene mineralization activity and were inactive for 30 to up to more than 100 days after the temperature shift phase; then they started to mineralize toluene and produce sulfide again, in considerably higher rates than the replicates incubated temporarily at 38°C (Figs 3B; S23BCD, S24BCD_Supplementary Data). In Wg38 setups, a transient shift to 12°C, 25°C, 45°C, or 60°C inhibited toluene mineralization, but the cultures began mineralizing toluene and producing sulfide within 30 days after the temperature had been shifted back to 38°C. Notably, one replicate of the 12°C-affected culture showed higher toluene mineralization and sulfide production rates than observed before, and the 60°C-affected replicates mineralized toluene in lower rates compared to the non- and lower temperature affected 38°C replicates and did not produce sulfide (Figs S16, S25ABCA, S26ABC_Supplementary Data).

Composition of toluene-mineralizing communities grown at different temperatures

Community composition of toluene-mineralizing communities enriched at constant temperature

Distinct communities developed with regard to the selected temperature (12–45°C) and field site (Zt, Wg), as shown by analysis of β diversity (Fig. 4). The individual replicates were very similar to each other, indicating a high selection pressure for the community.

Microbial community compositions for the cultures enriched at constant temperatures (Zt12, Zt20, Zt25, Wg12, Wg25, Wg38, Wg45) are shown at the genera (Fig. 5) and family (Fig. S27_Supplementary Data) levels. At 12°C, phylotypes affiliated to the genus *Desulfosporosinus* (*Desulfitobacteriaceae*) were highly abundant in Zt12 (59%–71%) and Wg12 (78–88%) replicates. *Desulfosporosinus* phylotypes were less abundant at 25°C (2%–5% in Zt25;

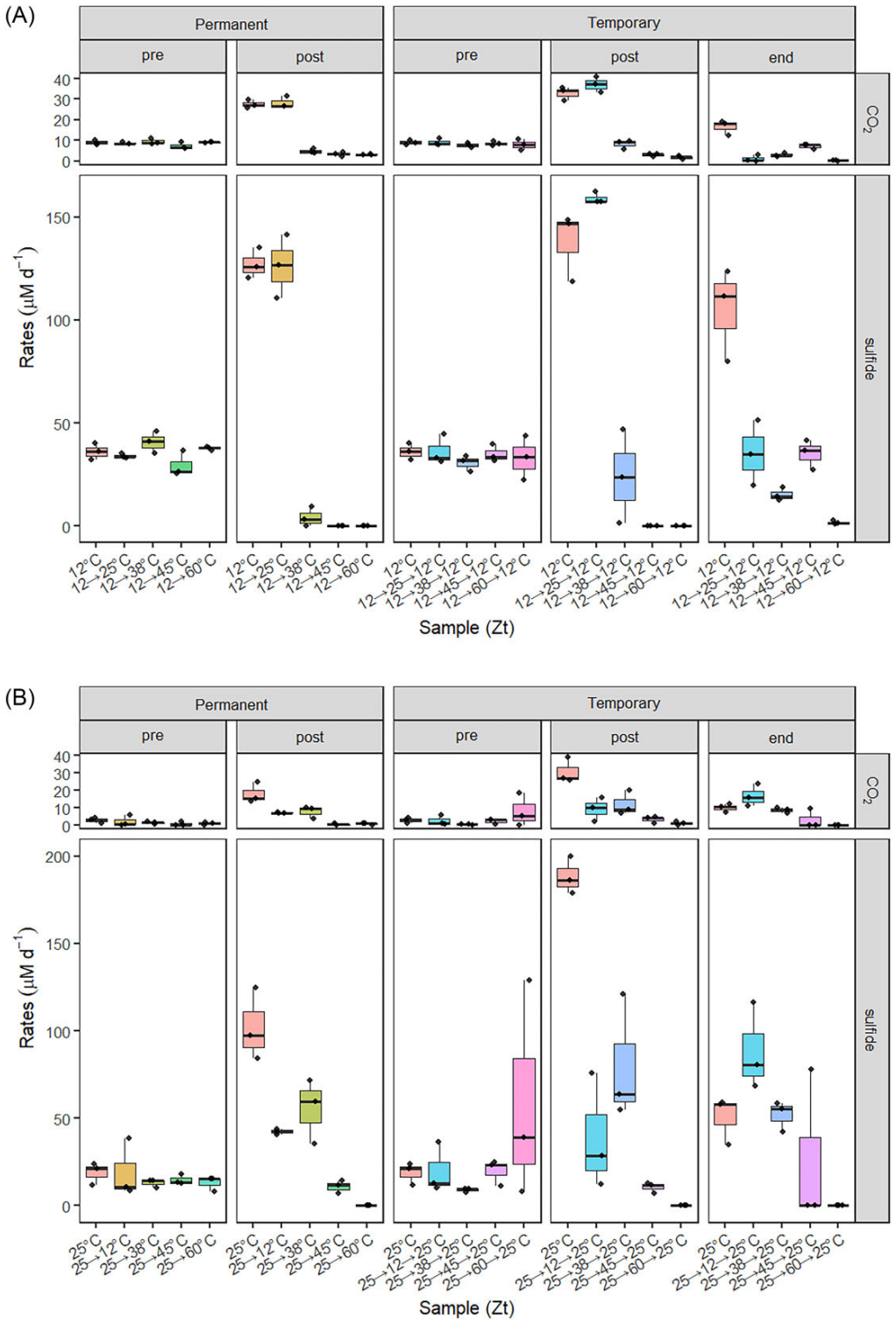


Figure 2. Rates of [^{13}C]-α-toluene mineralization and sulfide production of Zt12 (A) and Zz25 (B) microcosms after permanent or temporary temperature change. Data are average values of replicate microcosms ($n = 3$). 'Pre' summarizes initial rates at 25°C. 'Post' summarizes rates after temperature change. 'End' summarizes rates after the temperature was changed back to the original temperature. For comparison, rates for microcosms incubated at constant temperature (12°C in A or 25°C in B) were calculated as well for each period. Data of single replicates and lengths of time periods are presented in Figs S6–S9 and S16–S19 and Tables S9, S10, and S12 of the Supplementary Data. Rates in 12→25→12 'end' are low due to almost complete consumption of [^{13}C]-α-toluene.

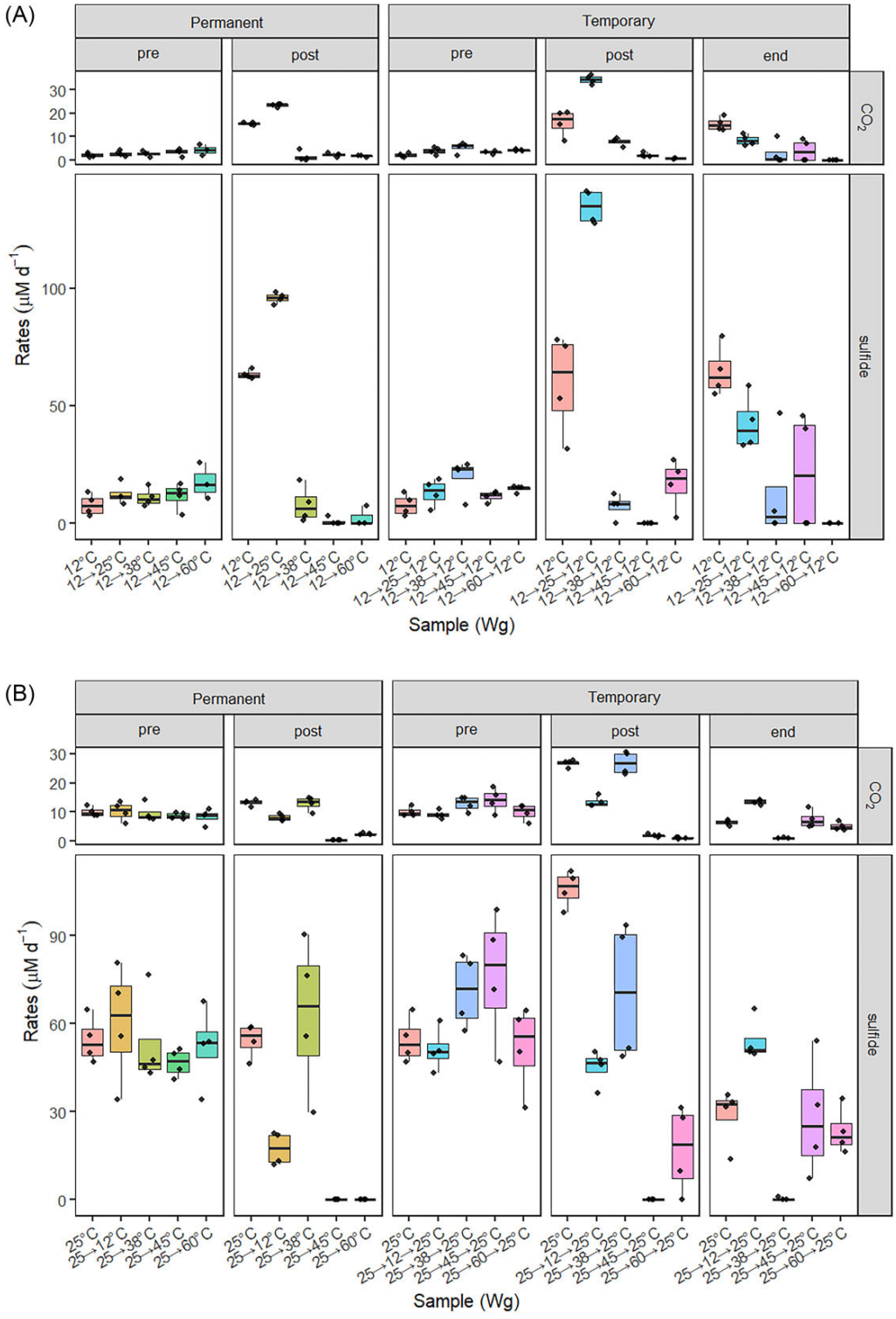


Figure 3. Rates of [^{13}C]-α-toluene mineralization and sulfide production of Wg12 (A) and Wg25 (B) microcosms after permanent or temporary temperature change. Data are average values of replicate microcosms ($n = 3$). 'Pre' summarizes initial rates at 12°C (A) or 25°C (B). 'Post' summarizes rates after temperature change. 'End' summarizes rates after the temperature was changed back to the original temperature. For comparison, rates for microcosms incubated at constant temperature (12°C in A or 25°C in B) were calculated as well for each period. Data of single replicates and lengths of time periods are presented in Figs S10–S13 and S20-–S23 and Tables S9, S11, and S13 of the Supplementary Data.

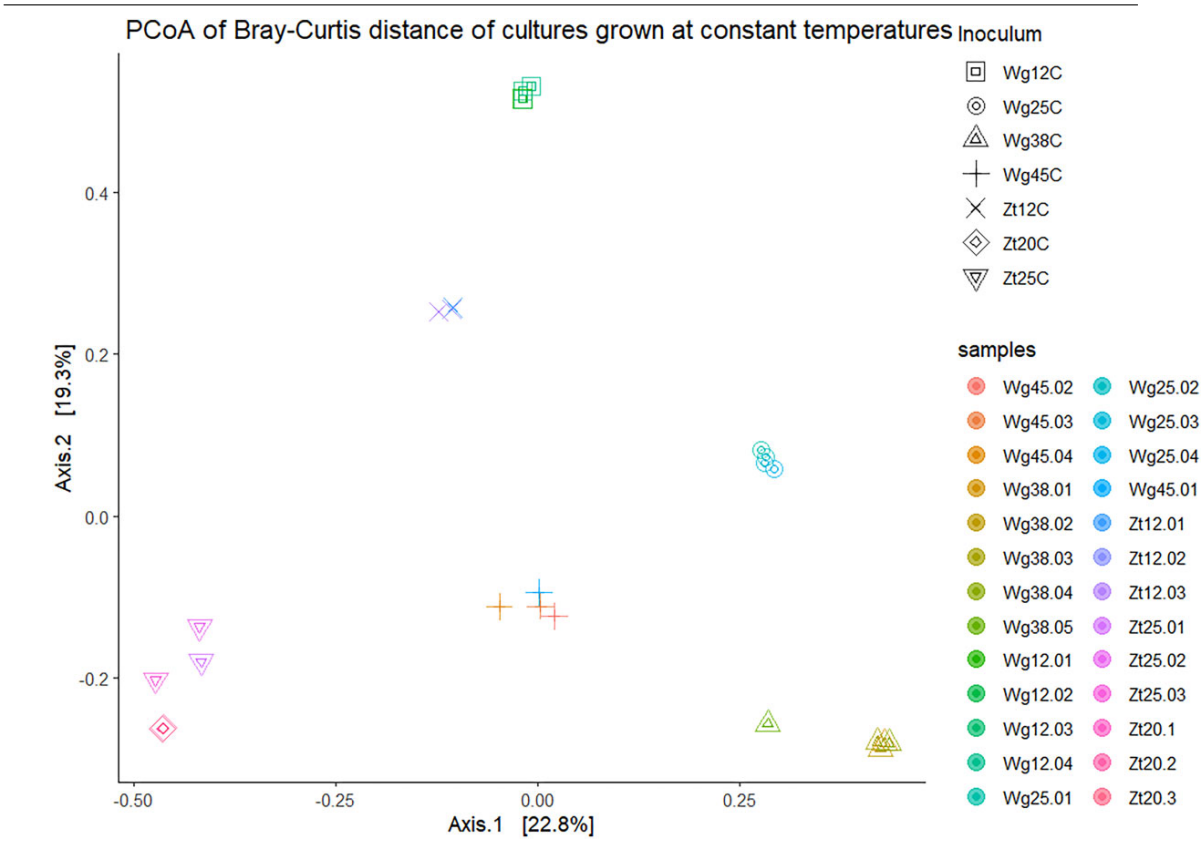


Figure 4. ß-diversity analysis according to BC dissimilarity of the cultures enriched with toluene and sulfate at various constant temperatures.

11%–17% in Wg25) and negligible at 38°C and 45°C (Wg38, Wg45). At 20°C (Zt20), *Desulfoprunum* (88–89%) dominated in all replicate cultures. At 25°C, *Desulfoprunum* was also enriched in Zt25 (23%–41%), alongside *Desulfovibrio* (10–18%), a *Desulfosarcinaceae* affiliated phylotype (7%–10%) and organisms related to *Spirochaetaceae* (11%–17%). In Wg25, other phylotypes dominated compared to Zt25: *Pelotomaculum* (6%–13%), *Desulfallas-Sporotomaculum* (6%–16%), *Anaerolineaceae* (7%–15%), and organisms related to the order OPB41 (14%–25%) and the family *Christensenellaceae* (5%–10%). Different *Anaerolineaceae* (12%–17%), *Pelotomaculum* (4%–12%), and organisms belonging to *Desulfallas-Sporotomaculum* (9%–15%) were also found in Wg38 in higher abundances; in addition to phylotypes affiliated to *Ammonificaceae* (5%–8%), *Gelria* (8%–12%), the order NRB23 (4%–12%), class D8A-2 of the phylum *Bacillota* (9%–12%), and especially *Proteiniphilum* (20%–27%). The communities of Wg45 replicates differed from the communities enriched at lower temperatures and comprised mainly phylotypes related to TTA-B15 of the order *Caldisericales* (6%–52%), *Desulfurispora* (39%–42%, two replicates), the order DTU014 of the class *Incertae Sedis* (8%–12%), and the class D8A-2 of the phylum *Bacillota* (12%–14%) that were enriched in Wg38, too.

Community compositions in experiments with permanently changed temperatures ('adaptation scenario')

Generally, the cultures that were permanently exposed to a different temperature (higher or lower) underwent considerable community changes at temperature shifts from 12°C (or 25°C) to 38°C, 45°C, or 60°C as shown in the Bray–Curtis (BC) dissimilarity plots (Figs S28–S32_Supplementary Data). BC plots also show a change in the composition in Wg38 setups shifted to 12°C, 25°C, and 45°C with all the exposures clustered separately from setups maintained at constant temperature (Fig. S32_Supplementary Data).

In Zt12, the abundance of *Desulfosporosinus* phylotypes decreased when exposed permanently to 25°C (12%–17%) or 38°C (30%–36%) when compared to 12°C (59%–71%); instead, *Sulfurovum* (7%–19% at 25°C and 28%–38% at 38%) and uncultured *Rikenellaceae* (43%–64% at 25°C) became dominant (Fig. S33_Supplementary Data). In Zt12 cultures shifted permanently to 45°C and 60°C and not any longer mineralizing toluene, *Desulfosporosinus* phylotypes remained in high numbers (24%–53% at 45°C, 80%–92% at 60°C). In Zt25 setups shifted to 12°C, *Desulfosporosinus* abundances increased (10%–31%), while *Desulfovibrio* (12%–20%) and *Desulfoprunum* (8%–32%) persisted (Fig. S34_Supplementary Data). In Zt25 cultures shifted permanently to 38°C, uncultured members of the *Desulfosarcinaceae* (28%–30%) and *Spirochaetaceae* (20%–24%) became dominant; however, Zt25 cultures permanently shifted to 45°C and 60°C still contained phylotypes belonging to the genera *Desulfoprunum*, *Desulfosporosinus*, *Desulfovibrio* in higher abundances (20%–24%), although toluene mineralization coupled to sulfate reduction was inhibited.

In Wg12 cultures shifted to 25°C, *Desulfosporosinus* phylotypes were slightly less dominant (59%–70%) than observed at 12°C (77%–88%), and almost disappeared during incubations at 38°C and 45°C (0.4–6%); here, *Pelotomaculum* (at 38°C), *Proteiniphilum* (38°C, 45°C), *Thiobacillus* (38°C, 45°C), *Pseudomonas* (38°C), and *Anaerolineaceae* (38°C, 45°C) became dominant (Fig. S35_Supplementary Data). When shifted to 60°C, *Desulfosporosinus* remained in high numbers (51%–84%). In Wg25 cultures shifted to 12°C, *Desulfosporosinus* became more abundant (43%–45%) but was almost absent in cultures shifted to 38°C or 45°C; here, *Desulfitibac-*

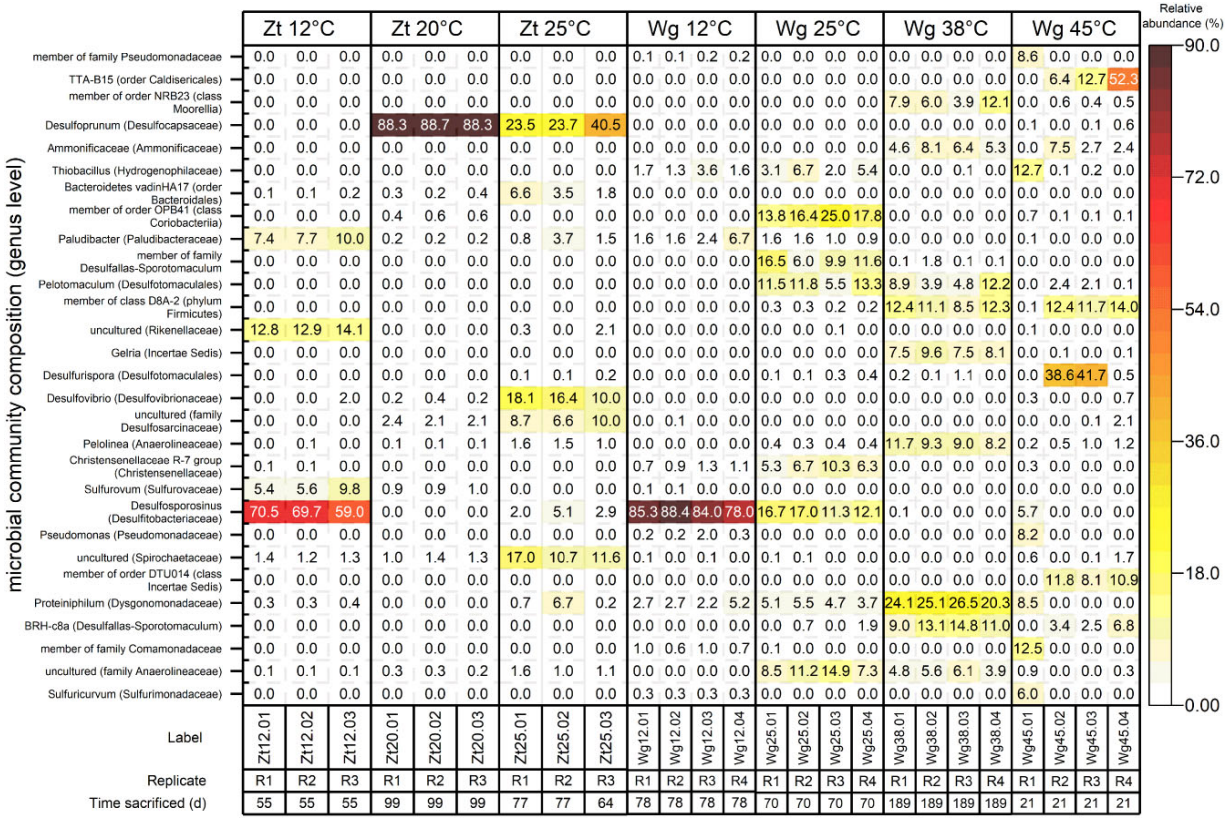


Figure 5. Microbial community composition of sulfate-reducing toluene-mineralizing Zt and Wg replicates incubated at different temperatures represented at the genus level and the corresponding family level in parentheses.

ter (at 38°C and especially at 45°C) and *Anaerolineaceae* (38°C, 45°C) became more abundant despite limited or absent toluene mineralization and sulfide production activity (Fig. S36_Supplementary Data).

In Wg38 cultures, members of the *Ammonificaceae* and the genus BRH-c8a (*Desulfallas-Sporotomaculum*) were still abundant when placed at 12°C, 45°C, or 60°C (Fig. S37_Supplementary Data), although setups shifted to these temperatures lacked toluene mineralization activity. *Proteiniphilum* were detected in higher numbers only at 12°C and 25°C, and members of the order NRB23 (class *Moorellia*) were enriched only at 45°C and 60°C.

Community compositions in temporary heat change experiments

Only communities enriched at 12°C (Zt12, Wg12) were considered with regard to community changes after a transient temperature shift. Both Wg and Zt communities transiently shifted to 25°C, 38°C, 45°C, or 60°C stopped to mineralize toluene temporarily at temperatures > 25°C but resumed to mineralize toluene in most replicates except those incubated temporarily at 60°C (Figs 3, S15, S16, S19_Supplementary Data). Effects of temperature on community structures were most pronounced at 45°C and 60°C, as shown by the BC dissimilarities (Figs S38, S39_Supplementary Data). Notably, *Desulfosporosinus* phylotypes that dominated the 12°C enrichments (Fig. 5) were still highly abundant in almost all of the replicates of the Zt and Wg series (Figs S42-S43_Supplementary Data). Phylotypes that had developed considerably (compared to non-affected 12°C communities, Fig. 5) during or after temperature change were affiliated to *Sulfurovum* (Zt12-38-12), uncultured *Rikenellaceae* (Zt12-25-12), uncultured *Spirochaetaceae* (Zt12-45-12), *Proteiniphilum* (Wg at 25°C, 38°C and 45°C) and *Gracilibacter* (Wg12-60-12), and phylotypes that got lost were affiliated to *Rikenellaceae* (Zt at 38°C, 45°C, 60°C) and *Proteiniphilum* (Wg 12–60-12) (Figs S40, S41_Supplementary Data).

Discussion

Toluene mineralization at sulfate-reducing conditions at different temperatures: key players and limits

Our data show that toluene was mineralized by aquifer communities from two different sites at sulfate-reducing conditions at temperatures between 12°C and 45°C. Enrichment cultures made from Weißandt-Gölzau sediment had a wider active temperature range (12°C–45°C) than cultures made of the Zeitz site material (12°C–25°C). In most cultures, the ratio of produced sulfide to mineralized toluene was close to the expected value of 4.1–4.5 based on equations (1) and (2), confirming the suitability of [^{13}C]-α-toluene in monitoring toluene assimilation and mineralization processes as previously suggested (Bombach et al. 2010). Data across all seven setups investigated in this study show that toluene-mineralizing communities were generally negatively impacted at 38°C: no enrichments could be established at this temperature for the Zt community, and the Wg community showed considerably reduced mineralization rates, indicating unfavorable growth conditions. This corresponds mainly to data from previous studies. Anoxically incubated microbial communities from hydrocarbon-contaminated soil degraded toluene at 22°C and

30°C, but not at 35°C and 40°C (Zeman et al. 2014). Most described sulfate-reducing toluene-mineralizing consortia have been cultivated at temperatures between 14°C and maximal 35°C (Beller et al. 1992, Müller et al. 2009, Bombach et al. 2010, Cupples 2011, Pilloni et al. 2011, Sun and Cupples 2012). Different mesophilic sulfate-reducing toluene-mineralizing pure strains for which the temperature range for growth was determined in more detail could not grow above 36°C (Harms et al. 1999, Morasch et al. 2004, Ommedal and Torsvik 2007), with the exception of *Desulfosporosinus youngiae* growing at up to 39°C (Lee et al. 2009). However, a community from Weißand-Gölzau sediment could be established mineralizing toluene at 45°C in high rates and observed for the first time at this temperature. This finding demonstrates the presence of toluene-mineralizing organisms adapted to temperatures above the usual upper temperature limit of mesophilic microorganisms and indicates a previously unknown potential of aquifer microbial communities to degrade hydrocarbons in the lower thermophilic temperature range. We can only speculate why this potential was obviously absent in material from the Zeitz site. The lag-phase for toluene mineralization at 45°C in the Wg sediment was extraordinary long (>1 year), which indicates that these organisms were very sparsely distributed in the used environmental samples. Similar patterns—irregular activity of organisms with certain degradation capacities in environmental samples—have been observed, e.g. for anaerobic benzene degraders (summarized in Vogt et al. 2011) or anaerobic MTBE degraders (Somsamak et al. 2001).

However, toluene-mineralizing sulfate-reducing cultures could not be established in our study at temperatures higher than 45°C, although such organisms have been once described to be present in deep hot oil reservoirs (Chen and Taylor 1997); hence, it is still an open question whether aquifer communities are able to mineralize aromatic hydrocarbons at thermophilic or hyperthermophilic conditions; e.g. in course of an HT-ATES operation.

Key toluene-mineralizing organisms at native temperature

At native groundwater temperature (12°C), phylotypes affiliated to the genus *Desulfosporosinus* became highly abundant in microbial communities from both tested sites (Zt and Wg). This indicates that *Desulfosporosinus* members metabolize toluene as a sole carbon source far more quickly or efficiently than other putative toluene-degrading taxa at this temperature, indicating a major role for toluene (and probably other aromatic hydrocarbons) mineralization in aquifers at psychrophilic (native) conditions. Previously described members of *Desulfosporosinus* grew optimally at temperatures between 25°C and 37°C but were also shown to be adapted to 12°C or even lower temperatures (Robertson et al. 2001, Ramamoorthy et al. 2006, Lee et al. 2009). *Desulfosporosinus* species have already been described as anaerobic sulfate-reducing toluene degraders: Besides the mentioned *Desulfosporosinus youngiae* (Lee et al. 2009), a few *Desulfosporosinus* phylotypes were identified or suggested as toluene-metabolizing organisms in sulfate-reducing microbial communities sampled from soil or aquifer sediments (Robertson et al. 2000, Alfreider and Vogt 2007, Winderl et al. 2010, Sun and Cupples 2012) or were shown to metabolize toluene in methanogenic communities (Winderl et al. 2010, Cupples 2011, Fowler et al. 2014, Laban et al. 2015).

Key toluene-mineralizing organisms at temperatures above native conditions (12°C)

Temperatures above 12°C caused strong shifts in the toluene-degrading sulfate-reducing microbial community (Fig. 4). At 20°C, *Desulfoprunum* phylotypes became dominant in addition to *Desulfosporosinus* phylotypes (Fig. 5). At 25°C, Zt and Wg cultures were dominated by phylotypes belonging to the taxa *Desulfoprunum*, *Desulfovibrio*, *Pelotomaculum*, *Desulfallas-Sporotomaculum*, and uncultured members of *Anaerolineaceae*. *Desulfoprunum* is a genus currently comprising one species, *Desulfoprunum benzolyticum*, a mesophilic sulfate reducer that grows between 20° and 37°C, using benzoic acid as carbon and energy source (Junghare and Schink 2015, Galushko and Kuever 2019). In recent studies, sessile *Desulfoprunum* phylotypes were shown to accumulate at the surface of toluene-amended adsorption material placed in anaerobic groundwater microcosms, indicating that they had metabolized toluene (Taylor et al. 2021, Johnson 2023). *Desulfovibrio* are non-spore forming mesophilic sulfate reducers growing optimally between 30°C and 38°C; these organisms have not yet been described for their capability to degrade toluene, although they are commonly found in marine or freshwater habitats at sulfate-reducing conditions (Pelz et al. 2001, Müller et al. 2009, Bombach et al. 2010, Jehmlich et al. 2010, Fowler et al. 2014, Kuever et al. 2015). *Desulfovibrio* species are physiologically versatile sulfate reducers with the ability to consume hydrogen very efficiently (Cord-Ruwisch et al. 1988, Bak and Pfennig 1991, Müller et al. 2009). It is postulated that they can utilize metabolites of toluene metabolism or dead biomass of toluene degraders at mesophilic conditions (Pelz et al. 2001, Bombach et al. 2010), which could also be their ecological niche in our study. *Pelotomaculum* are gram-positive mesophilic/thermophilic endospore formers capable of utilizing low molecular weight aromatics such as benzene (Rainey 2015, Dong et al. 2017). So far, only *Pelotomaculum* candidate BPL has the complete sulfate reduction pathway for benzene degradation; meanwhile, other phylotypes seemed unable to carry out dissimilatory sulfate reduction and couple syntrophically the fermentation of organic substrates with other organisms (Dong et al. 2017). It is believed that *Pelotomaculum* phylotypes initially attack and oxidize benzene at anoxic conditions, perhaps incompletely to acetate and dihydrogen, which are used by hydrogenotrophic and acetotrophic sulfate reducers (Phelps et al. 1998, Herrmann et al. 2010, Weelink et al. 2010, Gieg et al. 2014, Rainey 2015, Dong et al. 2017, Lueders 2017). *Desulfallas-Sporotomaculum* phylotypes are usually anaerobic endospore formers, fermenters or sulfate reducers, and thermophilic or moderately thermophilic; members of the genus *Desulfallas* were classified under the genus *Desulfotomaculum* until a few years ago (Watanabe et al. 2018). Aromatics-utilizing *Desulfallas-Sporotomaculum* phylotypes have been described (Braumann et al. 1998, Kuever et al. 1999).

Other phylotypes appearing in permanent temperature shift setups include uncultured members of the actinobacterial lineage OPB41and *Spirochaetaceae*. OPB41 bacteria are globally distributed in the subsurface, but only two isolates have been described yet; one of them, the mesophilic strain M08DHB^T, reduces anaerobically sulfur compounds using 3,4-dihydroxybenzoic acid as carbon and energy source (Khomyakova et al. 2022). Uncultured *Spirochaetaceae* were reported as necromass feeders (Dong et al. 2018).

Toluene-mineralizing community at 45°C

The toluene-metabolizing sulfate-reducing community enriched from Wg sediment at 45°C was considerably different to the communities enriched at 38°C and lower temperatures (Fig. 4), suggesting it was composed of toluene-metabolizing organisms specifically adapted to 45°C. Notably, different phylotypes had strongly accumulated (39%–52%) within replicates, affiliated to the genus *Desulfurispora* or the family TTA-B15 of the order *Caldisericales* (Fig. 5), indicating both may have used toluene as primary

carbon source. *Desulfurispora* belongs to the order *Desulfotomaculales* in which spore-forming, often thermophilic sulfate reducers are classified (Chuvochina et al. 2023) which are colonizing the subsurface down to great depths (Aüllo et al. 2013); a few organisms belonging to this group have been reported as toluene degraders, too (Morasch et al. 2004, Lee et al. 2009). The family TTA-B15 of the order *Caldisericales* is less good known. Previous studies revealed that some members are sulfate reducers (Martinez et al. 2019) or associated with methanogenic crude oil degradation at thermophilic conditions (Cheng et al. 2014).

To our knowledge, anaerobic toluene mineralization at 45°C has not been reported so far. Degradation of other petroleum compounds (mainly alkanes) at thermophilic conditions by sulfate reducers has been shown in several studies (Rueter et al. 1994, Kniemeyer et al. 2007, Gieg et al. 2010, Mbadinga et al. 2012, Zhou et al. 2012, Khelifi et al. 2014). Dissimilatory sulfate reduction with other electron donors has been recorded at even higher temperatures, peaking at 110°C, carried out by microorganisms from a hydrothermal vent where rates ranged between 19 and 61 µM SO_4^{2-} d^{-1} (Jørgensen et al. 1992). Sulfate-reducing laboratory cultures were successfully established at up to 85°C (Stetter et al. 1993, Marietou 2021). High sulfate reduction rates at temperatures > 50°C from up to 807 µmol L^{-1} to 5.3 ± 11 × 10^{-10} M s^{-1}(~45 792 µM d^{-1}) were also reported (Gieg et al. 2010, Bonte et al. 2013, Li et al. 2017). Nonetheless, the mentioned studies with regard to anaerobic hydrocarbon degradation and dissimilatory sulfate reduction were conducted using material from areas naturally exposed to high temperatures and, hence, the native communities developed into thermophilic communities over long time periods by natural succession.

Impacts of temperature changes on toluene mineralization

Prolonged HT-ATES applications will result in temporarily or permanently lasting strong increases of temperature ('heat shocks') in distinct aquifer areas, either directly from close proximity to the core or in the further outer layers experiencing an gradual temperature increase (Daniilidis et al. 2022). In our study, the temporary exposure of heat was restricted to fourteen days to partially simulate seasonal temperature changes serving as a temporary heat shock, while the permanent transfer simulated consequences of areas with prolonged high temperature storage. Generally, temperature exposures ≥ 38°C were critical since toluene mineralization was severely limited or completely inhibited. These limitations would justify why the upper thresholds for LT-ATES are kept at 25°C (Fleuchaus et al. 2018), especially if combined with bioremediation. Perpetual absent sulfate reduction and toluene mineralization due to prolonged permanent incubation (>150 d) at 60°C indicates the absence of thermotolerant or thermophilic sulfate-reducing toluene-mineralizing community members operating at ≥ 60°C in our study. Notably, toluene-mineralizing communities enriched at *in situ* temperatures (12°C) exposed temporarily to 60°C could not restore its toluene-mineralizing activity, in contrast to the same communities temporarily exposed to 45°C (Fig. 3); thus, the higher the temperature, the more detrimental to the toluene-mineralizing community. Many *Desulfosporosinus* phylotypes can form heat-resistant endospores (Hippe and Stackebrandt 2015) allowing them to survive hostile environmental conditions, but it seems the toluene-mineralizing *Desulfosporosinus* of our study did not possess this property, at least not in the time frame of our experiments. Prolonged cyclic exposure at higher temperatures could hence generally damage the degradation potential of microbial communities of shallow aquifers, although this has to be investigated further, only a few studies investigating this topic are available (Yadav et al. 2012, Saito et al. 2016, Bin Hudari et al. 2022).

Implications and outlook

This study highlights factors such as temperature limits, operational temperature, presence of thermophiles/thermotolerant or resilient 'native' communities in response to HT-ATES operations in hydrocarbon contaminated aquifers, using toluene as model contaminant and sulfate as electron acceptor. From a microbiological point of view, particularly the toluene (and generally aromatics hydrocarbon) mineralization potential above the temperature range of mesophilic organisms (≥45°C) is not understood but should in case of combining HT-ATES with bioremediation of hydrocarbon contaminated aquifers to be able to assess the consequences of the HT-ATES operation on the natural degradation potential of the aquifer. Hence, investigating the microbial community composition and remediation potential with regard to different temperatures should be addressed before trying to combine HT-ATES with bioremediation (Paliwal et al. 2012, Kaur et al. 2022). Currently, it remains unclear if anaerobic aromatics-degrading thermophiles or hyperthermophiles will be enriched by HT-ATES operations in shallow aquifers since these—as shown in our study—are seemingly absent or not cultivable at laboratory conditions at temperatures ≥ 60°C. Nevertheless, the enrichment of a specific community mineralizing toluene at 45°C from a shallow aquifer sediment sample that has never experienced temperatures above 15°C before hold some promise that aquifer sediments contain various different thermophiles having unexpected abilities and getting active at the right conditions and time; we are also aware that laboratory microcosm experiments can only provide limited evidence of processes that could take place *in situ* (Paliwal et al. 2012). Microcosms may differ from actual aquifers in substrate distribution and redox gradients, which can prevent a degradation potential observed in the laboratory from developing *in situ*. On the other hand, similar anaerobic hydrocarbon degradation reactions were shown to occur at laboratory, pilot and field scale (Fischer et al. 2009), demonstrating that processes observed in laboratory microcosms made of sediment samples may indeed reflect ongoing *in situ* processes.

Furthermore, studies must assess if continuous temperature change cycles can sustain activity, a resilient community and degradation potential, and if there will be a long-term thermal cycling fatigue. It is established that seasonal ATES can perturb the subsurface (Saito et al. 2016), but the effects of repeated heat exchange cycling at higher temperature thresholds as well as on nutrient availability and contaminants physicochemical characteristics need to be investigated (Head et al. 2014). For example, studies on organic carbon, total nitrogen or phosphorus changes in response to temperature have been described (Jiao et al. 2016, Geng et al. 2017, Gong et al. 2019). Future studies could incorporate multiple heat cycle experiments, or coupling different heat exchange scenarios with stable isotope probing to investigate the long-term effects of HT-ATES on ecosystem services and bioremediation. Looking forward, transitioning from LT-ATES to HT-ATES installation without compromising contaminant attenuation must be economically viable. Meanwhile, gaining societal 'acceptance' has to be conducted in parallel, perhaps by educating the masses of the long-term viability of HT-ATES and allaying concerns about 'microbial desertification'. Otherwise, alternatives including bioaugmentation and biostimulation that provides either the essential communities or parameters to promote remediation can be introduced.

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Author contributions

Mohammad Sufian Bin Hudari (Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization Writing – original draft), and Carsten Vogt (Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing).

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: None declared.

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Data availability

All data are available on request, sequencing data have been deposited in a public data base as mentioned in the text.

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Chapter 5.

Effect of temperature on microbial reductive dehalogenation of chlorinated ethenes: a review

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Effect of temperature on microbial reductive dehalogenation of chlorinated ethenes: a review

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One sentence summary: Temperature changes affect microbial dechlorination of chlorinated ethenes to a greater extent compared to competing and synergistic microbial processes such as fermentation, methanogenesis and sulfate reduction, however, data gaps prevent final conclusions.
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Abstract

Temperature is a key factor affecting microbial activity and ecology. An increase in temperature generally increases rates of microbial processes up to a certain threshold, above which rates decline rapidly. In the subsurface, temperature of groundwater is usually stable and related to the annual average temperature at the surface. However, anthropogenic activities related to the use of the subsurface, e.g. for thermal heat management, foremost heat storage, will affect the temperature of groundwater locally. This minireview intends to summarize the current knowledge on reductive dehalogenation activities of the chlorinated ethenes, common urban groundwater contaminants, at different temperatures. This includes an overview of activity and dehalogenation extent at different temperatures in laboratory isolates and enrichment cultures, the effect of shifts in temperature in micro- and mesocosm studies as well as observed biotransformation at different natural and induced temperatures at contaminated field sites. Furthermore, we address indirect effects on biotransformation, e.g. changes in fermentation, methanogenesis, and sulfate reduction as competing or synergetic microbial processes. Finally, we address the current gaps in knowledge regarding bioremediation of chlorinated ethenes, microbial community shifts, and bottlenecks for active combination with thermal energy storage, and necessities for bioaugmentation and/or natural repopulations after exposure to high temperature.

Keywords: organohalide respiration, reductive dechlorination, chlorinated ethenes, temperature, groundwater contamination

Introduction

Organohalides comprise a diverse group of chemicals, which are used in various industries globally. However, its widespread industrial application also serves as an entry point into the environment. In fact, organohalides such as chlorinated solvents are amongst the main contaminant groups that are commonly occurring in the urban groundwater environment; the majority of these are derived from anthropogenic sources (Rittmann et al. 2000). Some of them can be highly persistent and toxic and are, therefore, listed as priority groundwater contaminants in the European Union and the USA (https://www.epa.gov/dwreginfo/chemical-contaminant-rules) or in the Stockholm Convention on Persistent Organic Pollutants (Kalnins et al. 2019).

Chlorinated ethenes (CE), such as tetrachloroethene (PCE), trichloroethene (TCE), and the partially dechlorinated metabolites dichloroethene isomers (DCE), and vinyl chloride (VC) are of a major concern mainly due to their frequent occurrence in the environment (Squillace 2004, Carter 2008, Weatherill et al. 2014). While PCE and TCE originate from anthropogenic sources, *cis*-DCE and VC tend to be associated with biotic transformation *in situ* (Hartmans 1985, Vogel and McCarty 1985, Nijenhuis et al. 2007). Although PCE and TCE are used in a variety of industrial and commercial applications, such as industrial solvents or degreasing agents, improper usage or disposal resulting in accidental spillage or leakages are notably amongst the main sources of these contaminants into the subsurface (Bishop 1993).

Groundwater contaminations including organohalides and urban areas usually overlap due to urban-related industrial activities (Rivett et al. 2012). Hence, mitigation strategies must be incorporated within the scope of the subsurface urban groundwater management. PCE and TCE form dense nonaqueous phase liquids and tend to sink, and migrate through the permeable aquifers before reaching a nonpermeable zone, resulting in plumes that are usually long due to the low abiotic and biotic degradation rates (OSWER Directive 1999, Fiedler and Gilbert 2013). Given the persistence of PCE, TCE, as well as DCE, and VC in the environment and their toxicity to human health, ensuring the complete conversion of these CE to the relatively harmless ethene or mineralization to CO_2 is paramount (National Research Council 2000, Huang et al. 2014).

Natural attenuation (NA) is one common mitigation strategy for organic groundwater contaminants (OSWER Directive 1999). Both abiotic and biotic transformations of CE depend on a multitude of factors such pH, temperature, and presence of electron donors/acceptors that can mediate or hamper dechlorination (Zhuang and Pavlostathis 1995, Lai and Lo 2007, Shapiro 2017). Although NA can proceed abiotically, such reactions tend to be limited and usually occur alongside biotransformation by dechlorinating bacteria (Wiedemeier et al. 1998, Dolinova et al. 2017). For organohalides, which include PCE and TCE, organohalide respiration is the main mode of biotransformation in anoxic environments (Adrian and Löffler 2016, Dolinova et al. 2017). CE are

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used as an electron acceptor with hydrogen as electron donor provided by fermentation of substrates such as lactate and pyruvate, or direct oxidation of low-weight organic compounds (Schink and Stams 2006, Fiedler and Gilbert 2013). Anaerobic transformation of PCE and TCE generally progresses via sequential dechlorination to *cis*-DCE, one of the three isomers of DCE (*trans*-DCE and 1,1-dichloroethene are less common intermediates) and further dechlorination to VC before yielding ethene (Fig. 1; OSWER Directive 1999). Overall, more bacterial species are known to be capable of utilizing PCE and TCE as electron acceptors generating the end product DCE when compared with those that are capable of transforming DCE or VC (Fig. 1 and Table 1; Table S1, Supporting Information; Dolinova et al. 2017). Probably as a consequence of the low cell number and diversity of species possessing genes, which confer the ability to dechlorinate DCE or VC, or unsuitable environmental conditions for the biochemical reaction, these compounds are typically accumulated at sites with PCE and TCE contamination (Fiedler and Gilbert 2013, Dolinova et al. 2017). Meanwhile to date, only a few bacterial strains belonging to the genera *Dehalococcoides* (i.e. *Dehalococcoides mccartyi*) and '*Candidatus* Dehalogenimonas etheniformans' are known to dechlorinate PCE or TCE completely to ethene while others are only capable of partially dechlorinating PCE or TCE to either DCE or VC (Tables S1, Supporting Information).

Temperature is one of the crucial factors affecting microbial activity and ecology in the environment (Dettmer 2002, Fiedler and Gilbert 2013, Jesußek et al. 2013, Shapiro 2017), and temperature changes may affect rates of other processes e.g. nitrogen and carbon cycling significantly (Hantschel et al. 1995, Yvon-Durocher et al. 2010, Jung et al. 2011). Temperature can also influence rates of other processes such as fermentation or acetogenesis, which can indirectly support dechlorination by supplying electron donors (e.g. hydrogen) or carbon sources (e.g. acetate). On the contrary, temperature can enhance growth of microorganisms within the community, which compete with dechlorinating bacteria for the same electron donors and carbon sources, examples being methanogens or sulfate reducers (Smatlak 1996, Chambon et al. 2013, Wei et al. 2016). In general, there are limitations to which increasing temperatures can affect rates of biochemical reactions. Rates of processes driven by microbes are highest within a certain temperature range at the temperature optimum, beyond which rates usually rapidly decline (Mohr and Krawiec 1980, Huang et al. 2011). Once a threshold temperature is exceeded, irreversible damage can occur to cell components such as proteins and cell membranes resulting in the ceasing of activity. Similarly, below a certain temperature, activities will decrease significantly and stop (Mohr and Krawiec 1980, Huang et al. 2011).

Microbial growth and activity temperatures are characteristic for each microorganism, and microorganisms and microbial communities are able to tolerate temperature changes to a certain extent (Madigan et al. 2018). Typically, the growth range for psychrophiles, mesophiles, thermophiles, and hyperthermophiles can be roughly defined as 0–20°C, 8–48°C, 40–70°C, and 65–90°C, respectively (Madigan et al. 2018) and these microorganisms are usually adapted to temperature ranges of their habitat. However, some taxonomic groups within the Gram-positive bacteria develop spores resistant to extreme hot or cold temperatures, allowing them to survive long periods at unfavorable temperature conditions. Owing to the fact that most enrichment cultures and isolates have been obtained using material from 'mesophilic' habitats, most described cultures are actually mesophiles. While there have been attempts to establish dechlorinating cultures from materials from hot and cold environments these were mainly unsuccessful and are not in published literature (see section 'Effect of temperature on microbial reductive dehalogenation by microbial communities at laboratory and field conditions').

In general, the temperature of groundwater is stable and correlated to a given depth, becoming constantly higher in direction of the earth center (Goldscheider et al. 2006, Griebler and Lueders 2009); groundwater temperatures may only fluctuate annually in a relatively narrow range in shallow aquifers depending on factors such as annual seasonal average temperature changes at the surface and aquifer recharge (Benz et al. 2017, Moeck et al. 2020). It has also been observed to respond, albeit with some delay and at a small degree, to climate change (Menberg 2014, Menberg et al. 2014, Benz et al. 2017, Previati and Crosta 2021). Notably, other anthropogenic activities, including inputs from local heat sources (e.g. subways; Menberg et al. 2013), or the use of the subsurface, e.g. for thermal energy storage (Fleuchaus et al. 2018), can affect the temperature of groundwater locally in larger magnitudes. Currently, two main types of underground thermal energy storage systems (UTES) are implemented for heating and cooling of buildings (Fleuchaus et al. 2018): borehole thermal energy storage (BTES), where heat or cold is extracted from a volume of rock or soil, and aquifer thermal energy storage (ATES), from which heat or cold is extracted from groundwater whilst being pumped from one well to another (Fleuchaus et al. 2018, 2020). For ATES, national regulations require a balance in temperature over a certain period and limit the upper and lower temperatures of groundwater injected, e.g. between 5 and 25°C, however, other systems such as High Temperature ATES (HT-ATES), which introduce temperatures above 50°C do exist (Fleuchaus et al. 2018, 2020, Todorov et al. 2020).

Currently, there is a demand for application of ATES in urban areas, e.g. in redeveloped former brown field sites, which can be contaminated where this increased use of the subsurface for areas to fulfill high energy storage demands can overlap with organohalides-contaminated groundwater. For CE bioremediation, temperatures exceeding a certain maximum (~ 45°C) may halt or disrupt biotransformation resulting in accumulation of the toxic parent compounds PCE/TCE or its dechlorination reduction intermediates, DCE or VC. On the other hand, higher temperatures may trigger other effects such as increased rates of volatilization or abiotic CE degradation by reaction with iron compounds such as pyrite (FeS_2) or zero-valent iron (Costanza and Pennell 2007, Lai and Lo 2007, O'Carroll et al. 2013, Badin et al. 2016, Schaefer et al. 2017, Koproch et al. 2019). Since increasing temperatures in the subsurface are mainly due to anthropogenic activities (Menberg 2014), there is a need to revisit the current status of temperature-related research pertaining to the isolates, enrichment cultures, and *in situ* field sites experiments to allow conclusions on the effect of temperature on microbial dehalogenation and subsequent management of these temperature impacted contaminated sites.

In this review, we aim to summarize the current knowledge of the temperature range of microbial reductive CE dechlorination, compiling data for dechlorinating bacterial isolates (Table 1; Table S1, Supporting Information), microbial consortia, column studies, as well as field sites (Table S2, Supporting Information). We derived general upper and lower temperature limits for reductive CE dechlorination from the published studies, for both the direct dechlorination reaction as well as for processes indirectly affecting dechlorination. Finally, we outline research gaps and questions that need to be addressed for a better understanding on the effects

Table 1. Overview of optimal temperatures and temperature ranges for growth or activity of isolates and selected enrichment cultures capable of reductive dehalogenation of CE. For more information and details see Table S1 (Supporting Information). References for the corresponding strains/isolates are available in the Supporting Information (N.A. not available).

Microorganisms/culture	Strains	Temperature range	Final dechlorination products	References
Chloroflexota: *Dehalobium*, *Dehalogenimonas*, and *Dehalococcoides*				
'Dehalobium chlorocoercia'	DF-1	10–35°C	PCE → *trans*-DCE + *cis*-DCE	Wu et al. (2000, b, 2002a), Miller et al. (2005), Kittelmann and Friedrich (2008), May et al. (2008), May and Sowers (2016)
Dehalogenimonas	'*Candidatus* Dehalogenimonas etheniformans' *strain GP*	20–30°C	TCE → ethene	Yang et al. (2017b)
	Dehalogenimonas sp. strain WBC-2	RT	*trans*-DCE → VC	Jones et al. (2006), Manchester et al. (2012), Molenda et al. (2016)
Dehalococcoides mccartyi	195, BTF08, CBDB1, BAV1, VS, FL2, GT, MB, ANAS1, ANAS2, 11a, 11a5, DCMB5, CG1, CG4, CG5, 11G, CG3, SG1, GEO12, NIT01, IBARAKI, GY50, UCH007	15–35°C	PCE / TCE → ethene; some partial to *cis*-/*trans*-DCE or VC	Maymo-Gatell et al. (1997), Adrian et al. (2000), Richardson et al. (2002), Cupples et al. (2003), He et al. (2003, 2005), Muller et al. (2004), Sung et al. (2006a), Bunge et al. (2008), Cheng and He (2009), Cheng et al. (2010), Cichocka et al. (2010), Lee et al. (2011, 2013), Löffler et al. (2013), Wang et al. (2014b, 2019), Uchino et al. (2015), Yohda et al. (2015), Adrian and Löffler (2016), Ding et al. (2017, 2020), Ismaeil et al. (2017), Zhao and He (2019), Yan et al. (2021), Asai et al. (2022)
Bacillota: *Dehalobacter*, *Acetobacterium*, and *Desulfitobacterium*				
Dehalobacter	*Dehalobacter restrictus* PER-K23^T, TEA	10–37°C	PCE/TCE → *cis*-DCE	Holliger et al. (1993,1998), Wild et al. (1996), Nelson et al. (2014)
	Dehalobacter sp. strains 12DCB1, 13DCB1, TCP1	25–30°C	PCE → TCE; main product of strain 12DCB1 + *cis*-DCE	Nelson et al. (2011, 2014), Wang et al. (2014a)
Acetobacterium	WB1	30°C	PCE → TCE	Balch et al. (1977), Egli et al. (1988), Damborský (1999)
Desulfitobacterium	*Dsb. hafniense* strains DCB-2^T, PCE-S, TCE-1, TCP-A, Y51, G2, JH1 *Dsb. dehalogenans* strains PCE-1, JW/IU-DC1 *Dsb. metallireducens* strain 853-15A^T *Dsb.* sp. strains B31e3, KBC1, PR, Viet-1, PCP-1	13–45°C	PCE → DCE; some to TCE	Madsen and Licht (1992), Utkin et al. (1994), Bouchard et al. (1996), Christiansen and Ahring (1996), Gerritse et al. (1996), Loffler et al. (1997), Miller et al. (1997), Dennie et al. (1998), Damborský (1999), Gerritse et al. (1999), Tiedje (1999), Breitenstein et al. (2001), Suyama et al. (2001), Finneran et al. (2002), Shelobolina et al. (2003), Tront et al. (2006), Tsukagoshi et al. (2006), Villemur et al. (2006), Yoshida et al. 2007, Fletcher et al. (2008), Ding et al. (2014), Goris et al. (2015)
Thermodesulfobacteriota: *Desulfuromonas* and *Geobacter*				
Desulfuromonas	*Dsm. chloroethenica* strain TT4B *Dsm. michiganensis strains* BB1, BRS1	10–35°C	PCE/TCE to *cis*-DCE	Krumholz et al. (1996), Krumholz (1997), Sung et al. (2003)
Geobacter lovleyi	SZ^T, KB-1, LYY	10–40°C	PCE → *cis*-DCE	Duhamel and Edwards (2006), Sung et al. (2006b), Wagner et al. (2012), Lihl et al. (2019), Liang et al. (2021)
Campylobacterota: *Sulfurospirillum*				
Sulfurospirillum multivorans	Strain K	15–33°C	PCE/TCE→ *cis*-DCE	Scholz-Muramatsu et al. (1995)
Sulfurospirillum halorespirans	Strain PCE-M2	Opt: 25–30°C	PCE/TCE→ *cis*-DCE	Luijten (2003)
Sulfurospirillum sp.	Strains JPD-1, ACS $_{TCE}$, ACS $_{DCE}$	JPD-1: 1.5–40°C ACS $_{TCE}$: 21°C ACS $_{DCE}$: 21°C	PCE to TCE; strain ACS $_{TCE}$ PCE to *cis*-DCE; strains JPD-1 and ACS $_{DCE}$	Laanbroek et al. (1977), Pietari (2002), Yang et al. (2017a), Huo et al. (2020)
Actinomycetota: *Propionibacterium*				
Propionibacterium	*strains HK-1 and HK-3*	30°C	Dechlorination to ethene	Stackebrandt et al. (2006), Chang et al. (2011)

Abbreviations: CE; chloroethenes; DCE; dichloroethene; N.A.; not available; PCE; perchloroethene; RT; room temperature; T; temperature; TCE; trichloroethene; and VC; vinyl chloride.

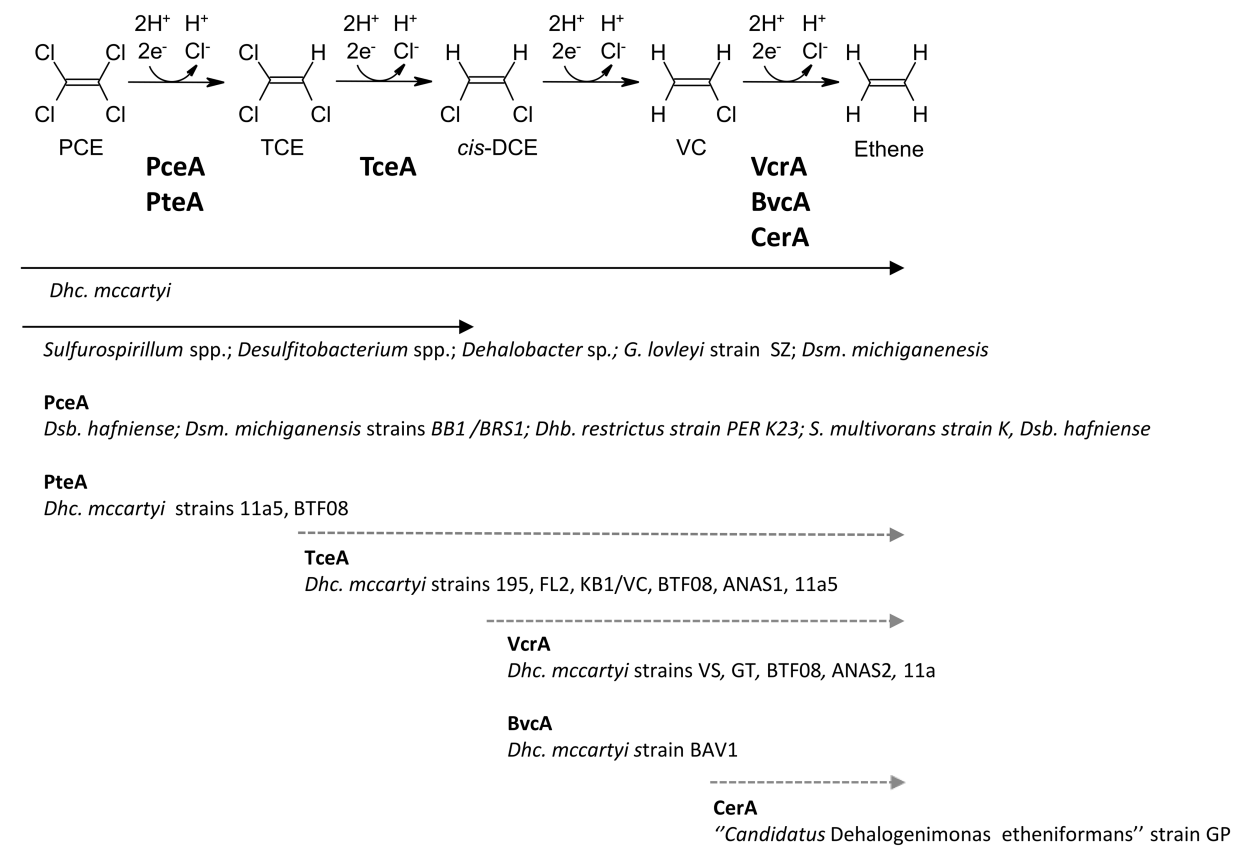


Figure 1. Overview of reductive dehalogenation of PCE; via TCE, DCE, and VC to ethene, with respective enzymes; PceA, PteA, TceA, VcrA, BvcA, and CerA and some of the reported microorganisms capable of the partial reaction.

of temperature on microbial dehalogenation and a combined application of ATES with bioremediation.

Temperature optima and growth ranges of organohalide-respiring bacterial isolates

Organohalide-respiring bacteria (OHRB) have been isolated from a variety of sources, many of which originated from chlorinated hydrocarbon contaminated sites from various continents and mainly coming from temperate climate zones (Table S1, Supporting Information). However, isolation was particularly targeted at obtaining isolates with dehalogenating capability for the purpose of further characterization; this process is usually related to availability of incubation temperatures in laboratories, e.g. room temperature (~20–25°C) or with a focus on the *in situ* temperature at the sampling sites. Hence, few studies investigating the effect of temperature shifts on the dechlorination potential of the isolates have been published. Notwithstanding that, not all the studies contain information on temperature range for growth, optimal temperature, or temperature at which dehalogenation was observed. While a number of the genera and strains have been substantially characterized [e.g. *Dehalococcoides (Dhc.) mccartyi*, *Desulfitobacterium (Dsb.) hafniense*, and *Sulfurospirillum multivorans*; Goris et al. 2015, Futagami and Furukawa 2016, Goris and Diekert 2016, Zinder 2016; also see Table S1 (Supporting Information)], information on temperature effects on dehalogenation potential as well as growth conditions seems to be unavailable for a significant number of the strains (Table S1, Supporting Information). Overall, not much has been described for the specific temperature-related limitations towards dehalogenation, which would be an important aspect for bioremediation purposes, and for assembling dehalogenating consortia for bioaugmentation applications.

According to our current knowledge, OHRB are distributed across several phyla: Chloroflexota, Bacillota, Thermodesulfobacteriota, and Campylobacterota. Amongst these, most OHRB capable of dechlorinating CE that have been isolated so far belong to the two genera: *Dehalococcoides* (phylum: Chloroflexota), where at least 20 strains have been reported affiliated with *Dhc. mccartyi* (Table S1, Supporting Information); *Desulfitobacterium* (phylum: Bacillota) with at least seven strains reported for *Dsb. hafniense*. These genera are amongst others described in the following Phylum subsections, with a focus on those capable of organohalide respiration using CE as electron acceptors.

Chloroflexota: within the phylum Chloroflexota, at least three genera with dehalogenating activity have been described: *Dehalogenimonas* and *Dehalococcoides and Dehalobium*. So far, only one isolate has been reported for the genus '*Dehalobium*', assigned *Dehalobium chlorocoercia* strain DF-1 (May and Sowers 2016, Oren and Garrity 2022). In addition to its ability to selectively dechlorinate certain chlorinated polychlorobiphenyls (PCBs) and highly chlorinated benzenes, *D. chlorocoercia* strain DF-1 can also dechlorinate PCE and TCE to yield, albeit incompletely, higher amounts of *trans*-DCE than *cis*-DCE, when supplemented with H_2 or formate as an electron donor (Miller et al. 2005, May et al. 2008). *Dehalobium chlorocoercia* strain DF-1 grows optimally at 30–33°C, while no growth was reported at 10°C or 35°C (Wu et al. 2000, 2002a, b,

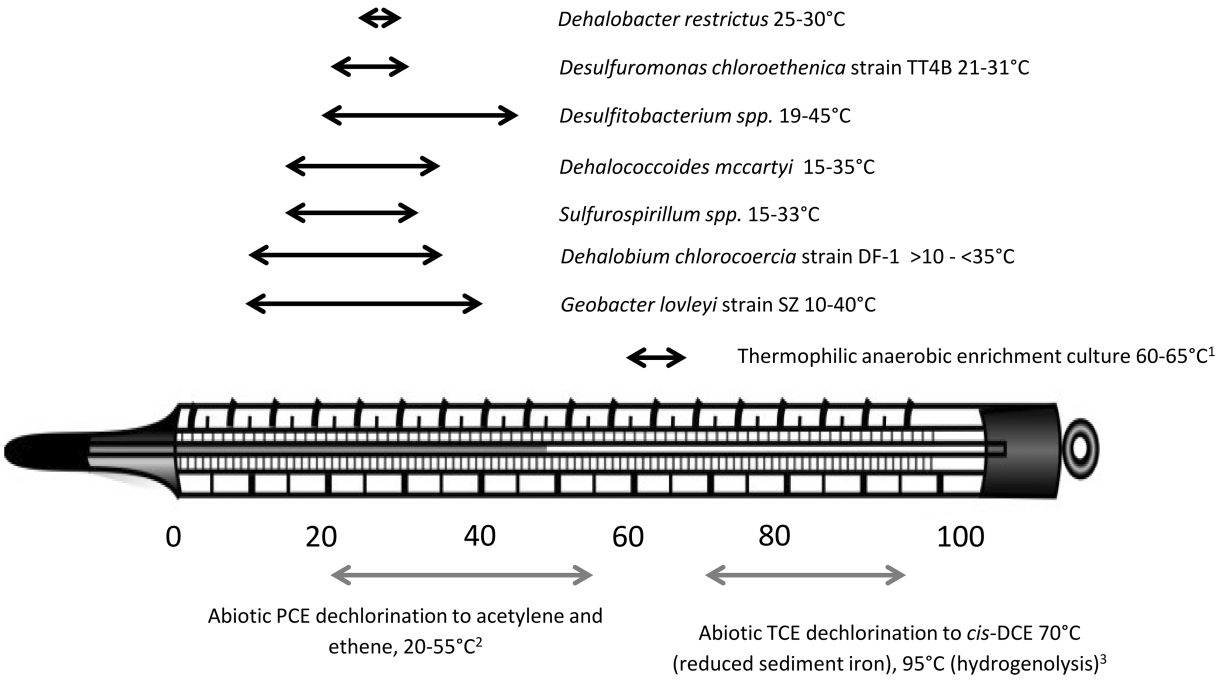


Figure 2. Overview of the temperature activity range for a select group of CE-dechlorinating bacteria and the range of temperature for some examples of abiotic dechlorination processes of the CE (1) Kengen et al. (1999), (2) Schaefer et al. (2017), and (3) Costanza and Pennell (2007) and Truex et al. (2007). References for the temperature range of microorganisms can be found in Table 1 and Table S1 (Supporting Information).

Miller et al. 2005, Kittelmann and Friedrich 2008, May et al. 2008). Within the genus *Dehalogenimonas (Dhgm.)*, the two well described species, *Dhgm. lykanthroporepellens* and *Dhgm. alkenigignens* were only capable of dehalogenating a variety of organohalides such as 1,2-dichloroethane and 1,1,2-trichloroethane but not any of the CE (Moe et al. 2009, Yan et al. 2009, Bowman et al. 2013). However, at least two strains have since been reported: '*Candidatus* Dehalogenimonas etheniformans' and *Dehalogenimonas* sp. strain WBC-2, which could dechlorinate one the CE (Table S1, Supporting Information). '*Candidatus* Dehalogenimonas etheniformans' strain GP, which dechlorinates TCE, DCE, and VC grows between 20 and 30°C with hydrogen (Yang et al. 2017b). *Dehalogenimonas sp.* strain WBC-2, which was enriched from the West Branch Canal Creek (WBC-2) culture dechlorinates mainly *trans*-DCE to VC at room temperature (Jones et al. 2006, Manchester et al. 2012, Molenda et al. 2016).

Most bacterial isolates within the phylum Chloroflexota capable of organohalide respiration belong to the genus *Dehalococcoides* (Table S1, Supporting Information). Many *Dhc.* strains can dechlorinate CE, some of them completely to ethene. *Dehalococcoides mccartyi* strain 195^T (formerly known as *Dhc. ethenogenes*) is the type strain for this genus (Maymo-Gatell et al. 1997, McMurdie et al. 2009, Löffler et al. 2013), dechlorinate PCE and TCE to VC, and cometabolically to ethene, with hydrogen as the electron donor (Maymo-Gatell et al. 1997, 1999, Yan et al. 2021). In general, *Dhc. mccartyi* strains are grown under mesophilic conditions with most strains cultivable between 15 and 35°C (Fig. 2; Maymo-Gatell et al. 1997, Rosner et al. 1997, He et al. 2003, Muller et al. 2004, Sung et al. 2006a, Cheng and He 2009, Löffler et al. 2013, Adrian and Löffler 2016). *Dhc. mccartyi* strain 195^T has an optimum growth temperature of 35°C, while above this dechlorination activity only persisted temporarily, and no dechlorination occurred at 40°C (Maymo-Gatell et al. 1997, McMurdie et al. 2009, Löffler et al. 2013); strains CBDB1, BAV1, VS, FL2, and GT grow at optimum temperatures between 25 and 30°C (Adrian et al. 1998, 2000, He et al. 2003, Sung et al. 2006a, Löffler et al. 2013, Adrian and Löffler 2016). Other strains such as MB, ANAS1, ANAS2, 11a, and DCMB5 possess optimum temperatures of 30°C (Richardson et al. 2002, Cheng and He 2009, Lee et al. 2011, Low et al. 2015).

Bacillota: CE dehalogenating species of this phylum belong to the genera *Dehalobacter (Dhb.)*, *Desulfitobacterium* (*Dsb.*), *Sporomusa*, and *Acetobacterium* (Table S1, Supporting Information). A total of two strains of *Dhb. restrictus*, strain PER-K23^T and strain TEA, are non spore-forming and have optimum growth temperatures of about 30°C (PER-K23: 25–30°C with no growth above 35°C; Holliger et al. 1993, 1998, Wild et al. 1996, Nelson et al. 2014). Meanwhile, several strains have been described within the genus *Dehalobacter,* such as strains 12DCB1, 13DCB1, and TCP1 (Table S1, Supporting Information). All *Dhb.* strains reported here have growth temperatures of 25–30°C (Holliger et al. 1993, 1998, Yoshida et al. 2009, Nelson et al. 2011, 2014, Wang et al. 2014a). Strains belonging to the genus *Desulfitobacterium* (*Dsb.*) are facultative OHRB, which enable them to use a wide spectrum of electron donors and acceptors. Within this genus, several strains are capable of dechlorinating at least one of the CE, comprising the species *Dsb. hafniense*, *Dsb. dehalogenans*, and *Dsb. metallireducens* including several which are categorized under *Dsb.* spp. (Table S1, Supporting Information). *Dsb. hafniense* strains DCB-2 (the type strain; Madsen and Licht 1992, Christiansen and Ahring 1996) and TCP-A can dechlorinate mainly PCE but not TCE (Breitenstein et al. 2001), while strains TCE-1 and PCE-S can utilize both PCE and TCE (Miller et al. 1997, Gerritse et al. 1999, Milliken et al. 2004, Ye et al. 2010, Goris et al. 2015). Temperature range of growth is between 13 and 45°C, varying between strains (Table S1, Supporting Information). *Dsb. hafniense* DCB-2^T was cultivated at 37°C (Madsen and Licht

1992, Christiansen and Ahring 1996), while *Dsb. dehalogenans* PCE-1 grows between 19 and 42°C (optimum: 34–37°C). *Dsb. metallireducens* strain 853-15A^T grows between 20 and 37°C, and no growth was observed at 4, 15, or 50°C (Finneran et al. 2002). Some strains can form spores, suggesting their capability to survive at higher temperatures, such as *Dsb. hafniense* strains DCB-2^T and PCE-S, and *Dsb.* sp. strain B31e3 are spore-forming (Madsen and Licht 1992, Christiansen and Ahring 1996, Löffler et al. 1996, Sanford et al. 1996, Kunapuli et al. 2010), while others such as *Dsb. hafniense* strain JH1, *Dsb. dehalogenans* strain JW/IU-DC1^T, and *Dsb. metallireducens* strain 853-15A^T are not spore-forming (Utkin et al. 1994, Finneran et al. 2002).

Thermodesulfobacteriota: to date, two several bacterial genera within this phylum were capable of reductive dehalogenation: *Desulfuromonas* and *Geobacter*. The genus *Desulfuromonas* (*Dsm.*) comprises two species with dehalogenating capabilities namely *Dsm. chloroethenica* and *Dsm. michiganensis*. *Dsm. chloroethenica* strain TT4B utilizes PCE and TCE as electron acceptors in the presence of the electron donors acetate or pyruvate, to yield *cis*-DCE (Krumholz et al. 1996, Krumholz 1997, Sung et al. 2003). This non spore-forming strain has a growth range of 21–31°C (optimum: 30°C) and did not dechlorinate at 16 or 35°C (Krumholz et al. 1996, Krumholz, 1997). *Dsm. michiganensis* strains BB1 and BRS1 are capable of dechlorinating PCE and TCE as electron acceptors (Sung et al. 2003), with a growth range between 10 and 35°C and an optimum growth temperature of 25°C (Table S1, Supporting Information). The second genus, *Geobacter* includes several *Geobacter lovleyi* strains (SZ, KB-1, and LYY), which can utilize PCE and/or TCE as electron acceptor, with *cis*-DCE as a major end product (Table S1, Supporting Information). *Geobacter lovleyi* strain SZ, the type strain, grows between 10 and 40°C (optimum: 35°C), does not form spores, and uses pyruvate, acetate, or hydrogen as electron donors (Sung et al. 2006b, Wagner et al. 2012), while *G. lovleyi* strains KB-1 and LYY grow at 22–25°C and 30°C, respectively (Duhamel and Edwards 2006, Lihl et al. 2019, Liang et al. 2021).

Campylobacterota: *Sulfurospirillum* (formerly *Dehalospirillum*) is the only known dehalogenating genus within this phylum comprising mainly *S. multivorans* and *S. halorespirans* (Table S1, Supporting Information). A number of these strains are mesophilic, growing between 20 and 30°C (Table 1; Table S1, Supporting Information) and are capable of metabolically dechlorinating PCE or TCE to *cis*-DCE (e.g. *S. multivorans* strain K, *S. halorespirans* strain PCE-M2, and *Sulfurospirillum*. sp. strain JPD-1; Laanbroek et al. 1977, Scholz-Muramatsu et al. 1995, Campbell et al. 2001, Pietari 2002, Luijten 2003). Spore formation was not described. One of the best described species, *S. multivorans* (formerly *Dehalospirillum multivorans* strain K), is able to dechlorinate PCE and TCE to *cis*-DCE, with electron donors such as hydrogen, formate, or pyruvate, grows between 15 and 33°C but optimally at 28–30°C and does not grow above 37°C (Scholz-Muramatsu et al. 1995). There are also several strains within the *Sulfurospirillum* sp. (strains JPD-1, ACS $_{TCE}$, and ACE$_{DCE}$) and *S. carboxydovorans* (strain MV) with optimal growth temperatures within the mesophilic growth range (~21–30°C; Table S1, Supporting Information; Laanbroek et al. 1977, Campbell et al. 2001, Pietari 2002, Jensen and Finster 2005, Yang et al. 2017a, Huo et al. 2020).

Reductive dehalogenation at thermophilic conditions

Overall, there is a lack of reports describing reductive dehalogenation processes by microbial isolates at thermophilic conditions (above 45°C; Table S1, Supporting Information), suggesting that higher temperatures may hamper reductive dehalogenation. However, it should be noted that enrichment and isolation of dehalogenating bacteria has generally been done at 20–30°C, thus resulting in isolates adapted to these temperatures. To our knowledge, there are no reports about enrichment of thermophilic dehalogenating microbes from natural hot habitats such as volcanic areas, hot springs, oil reservoirs, or deep aquifers. It is possible that since these habitats do not contain halogenated organics in higher concentrations, and were hence not the main focus in investigations. Nevertheless, it is an open question whether these hot natural habitats are inhabited by thermophilic dehalogenating microorganisms. Notably, the solubility of CE is slightly decreasing from 20 to 40°C, but again increasing at temperatures above 40°C (Koproch et al. 2019), hence reductive dehalogenation reactions should not be limited by physical–chemical constraints at higher temperatures.

Kengen et al. (1999) describe a thermophilic anaerobic enrichment culture from PCE-polluted sediment from Rotterdam harbour containing relatives of *Dhb. restrictus* and *Desulfotomaculum thermosapovorans*, capable of dechlorinating PCE to *cis*-DCE at 65°C (Table 1), indicating that microbial reductive dehalogenation, which was likely attributed to *Dhb. restrictus*, is principally feasible at thermophilic conditions. Therefore, more studies are needed to be done at higher temperatures to better understand the limits at which microbial growth and dechlorinating ability can persist, as well as remain resilient during a temporary period of high temperature exposure.

Nevertheless, strains possessing a temperature range of ≥ 25°C for growth are probably more tolerant to environmental temperature fluctuations, e.g. *G. lovleyi* strain SZ (10–40°C), *Dsb. frappieri* strain PCP-1 (15–45°C), and *Dsb. hafniense* strain PCE-S, which has a growth range between 20 and 45°C (optimal: 37–38°C) respectively (Table S1, Supporting Information). At contaminated sites exposed to elevated temperatures, a succession of microbial communities may result in a dominance of spore formers; however, this is expected to lead to partial dechlorination to DCE (Keynan et al. 1964, Berg and Sandine 1970, O'Sullivan et al. 2015). Spores which are usually resistant to high (boiling) temperatures confer the members of this respective community with the ability to reestablish themselves post heat exposure (O'Sullivan et al. 2015, Chan et al. 2021). For example, the growth and activity of the spore-forming *Dsb. chlororespirans* strain Co23 recovered 1 week post incubation at 80°C, after being returned to 30°C (Sanford et al. 1996).

Effect of temperature on microbial reductive dehalogenation by microbial communities at laboratory and field conditions

The effect of temperature shifts on microbial dehalogenation has been investigated mainly in the context of thermal treatment of contaminated field sites, as well as, more recently, with the combination of ATES and bioremediation in mind (Table S2, Supporting Information). However, only few studies have been published systematically investigating the effect of temperature.

Over the years, several consortia have been established including BDI, OW, KB-1, WBC-2, and SDC-9 (Table S2, Supporting Information). Consortium BDI comprises *Dhc. mccartyi* strains BAV1, FL2, and GT with a cultivation temperature of 24°C; ethene was produced at 30°C, but VC accumulated at 35 and 40°C, however, cultivation at 45°C resulted in a loss of dechlorination activity

(Fletcher et al. 2011a). Consortium OW was cultivated at 30°C and consists of multiple *Dehalococcoides* strains as well as *Dehalobacter*, *Geobacter*, and *Sulfurospirillum* (Fletcher et al. 2011b). Similar to BDI, dechlorination activity was lost at 45°C while VC accumulated at 35 or 40°C (Fletcher et al. 2011a). Consortium KB-1, which consists of *Dehalococcoides* and phylotypes affiliated to the genus *Geobacter*, was cultivated at room temperature and completely dechlorinated TCE between 10 and 30°C, but it accumulated *cis*-DCE outside the mentioned temperature range; no dechlorination activity was observed at 50 and 60°C (Friis et al. 2007b). Consortium SDC-9, which comprised *Dehalococcoccoides* and *Desulfitobacterium* as the two most abundant genera, were cultivated between 21 and 30°C, but have been reported to dechlorinate at 5°C and up to 40°C (Vainberg et al. 2009, Vainberg and Steffan 2014, Kucharzyk et al. 2020). Consortia WBC-2 and DehaloR 2 were cultivated at 19°C and 30°C, respectively, however, no further range of temperatures were described (Table S2, Supporting Information; Jones et al. 2006, Lee and Lee 2016).

The lowest temperature at which reductive dehalogenation of TCE to DCE and VC was reported is 4°C, proven by formation of [^{14}C]-VC and [^{14}C]-*cis*-DCE from [^{14}C]-TCE, in microcosms prepared with contaminated sediments from Alaska (Bradley et al. 2005). Incubations with consortium KB-1 below 10 or 15°C, with lactate or propionate as electron donor, respectively, in batch culture, or at 15°C in a column system, accumulated *cis*-DCE, suggesting comparatively lower activity of *Dhc. mccartyi* at lower temperature (Friis et al. 2007b, Marcet et al. 2018b). However, it should be noted that these experiments were done with consortium KB-1, which was continuously cultivated at room temperature, probably leading to a loss of slow-growing reductive dechlorinators adapted to lower temperatures in the original inoculum. Meanwhile, in other studies with consortium KB-1, complete reductive dechlorination to ethene was observed in laboratory studies at temperatures as low as 4°C with lactate (Heimann et al. 2007) and up to 43°C in KB-1 bioaugmented column experiments (Marcet et al. 2018a; Table S2, Supporting Information). In these studies, an increase in temperature resulted in a complete dechlorination to ethene. Contrastingly, a laboratory study by Nagymate et al. (2020) observed dehalogenation of TCE to ethene with corresponding presence of *Dhc. mccartyi* at 8°C (Nagymate et al. 2020), stressing the importance of the temperature used for cultivation, especially in the case of bioaugmentation.

However, it is important to note that the upper limits differ for different systems, could instead hamper complete dechlorination past *cis*-DCE. For example, the upper limits for the dechlorination of PCE to ethene was 43°C in a column system (Marcet et al. 2018b), while in another study batch cultures with the same KB-1 consortium an upper limit of 30°C was reported (Friis et al. 2007b, Heimann et al. 2007). The highest currently reported temperature for dechlorination of PCE to *cis*-DCE is 65°C, by a thermophilic anaerobic enrichment culture containing relatives of *Dehalobacter restrictus* and *Desulfotomaculum thermosapovorans* (Kengen et al. 1999; Table S2, Supporting Information).

With that, it seems that complete dechlorination to ethene is more temperature sensitive when compared to incomplete dechlorination. However, there are many studies that do not provide information on temperature ranges and/or its effects on reductive dechlorination (Table S2, Supporting Information). Regardless, temperature ranges reported in the laboratory studies do not necessarily reflect the potential temperature range for dechlorination present in natural systems, since the diversity of dechlorinators are likely to be broader than currently known. The diversity in the temperature optima and ranges of the consortium members enable the consortium to adjust to the temperature whilst performing their dechlorination function especially during bioaugmentation. This may be useful in the event where different members of the consortium (each with different growth temperature ranges and optima), which are either involved in different steps of the reductive dechlorination, or contribute indirectly either via fermentation of substrates to supply carbon source and electron donor, can carry out the steps when temperatures are non-inhibitory.

A majority of the enrichment cultures were cultivated under mesophilic growth conditions such as KB-1 (room temperature) and OW (30°C; Friis et al. 2007b, Fletcher et al. 2011a; Table S2, Supporting Information). However, temperature-influenced loss of dechlorination activity could cause the accumulation of the toxic *cis*-DCE and VC outside the optimal temperature window (Fletcher et al. 2011b).

In a study done by our lab, which investigated dechlorination of TCE in CE-contaminated sediment in the temperature range between 10 and 60°C, a higher accumulation of VC at 30°C and *cis*-DCE at 40°C was observed when compared to replicates incubated at 10°C and 20°C where the major product was ethene (unpublished). In general these results fit with published results, the dechlorination to ethene occurred in the presence of *Dehalococcoidia* related phylotypes in experiments between 10 and 30°C, while this was absent at 40°C (Ni et al. 2015, Zhang et al. 2015, Yamazaki et al. 2020).

To our knowledge, at least two studies of tests directly at field sites have been published. A field pilot test in the Czech Republic was performed in which the groundwater at the contaminated field site was heated from the original temperature of 12–13°C to 35–40°C and amended with whey as electron donor (Nemecek et al. 2018). Ethene and methane were observed as products after heating and a rapid increase of the number of organohalide-respiring genera (*Dehalococcoides* and *Dehalobacter*) and reductive dehalogenase genes (*vcrA* and *bvcA*) was observed. Another study tested high temperature heating of groundwater from 10 up to 70°C at the Fort Lewis field site (Truex et al. 2007). While at lower temperatures *cis*-DCE was observed as a product from presumably biological dehalogenation, ethene, and acetylene were observed at higher temperatures, likely a result from abiotic dehalogenation catalysed by the reduced iron in the sediments.

Nevertheless, several studies have also discussed the role of the microbial community following a period of more drastic temperature fluctuations from thermal treatments, e.g. electrical resistance heating (> 100°C) and steam injection (between 100 and 120°C; Friis 2006a, Friis et al. 2006). Friis et al. (2006) also mentioned the role of the temperature following thermal treatment in predicting the biological remediation potential since this temperature can either promote or inhibit dechlorination activity (Friis 2006b). For example, in the Danish site where steam injection was applied followed by cooling, temperatures of 30–50°C were reported 1.5 months after completion (Friis 2006b, Friis et al. 2007a), whereas a longer duration was needed in the Fort Lewis site (> 100 d) before temperatures below 50°C were measured. Thus, these prolonged cooling times would require long periods before temperatures allow for growth of native or bioaugmented microbial communities and recovery of dechlorination activity. Another field study involved an analysis of a post-remediated PCE-contaminated site in Rødekro, southern Denmark, which underwent thermal remediation via steam injection in 2006. In their study, conducted 11 years after remediation, samples were obtained from wells drilled along the plume flowline to investigate parameters such as redox potential, CE concentrations, and iso-

topic compositions as well as qPCR for quantification of specific organohalide-respiring genera and reductive dehalogenase genes and microbial community analysis (Murray et al. 2019). Murray et al. (2019) observed the recovery of microbial PCE and *cis*-DCE dechlorination in the previously thermally remediated section of the field site, stimulated by the release of dissolved organic carbon (DOC) during steam injection. However, the investigations did not specifically ascertain if the recovery of dehalogenation activity was related to a microbial community surviving heat treatment or an inoculation from by-passing groundwater, which did not experience thermal remediation. The lack of information on the site's recovery potential following heat treatment further highlights the uncertainty towards the resumption of biotransformation activity in ATES-related heat storage cycles. Future studies should, therefore, elucidate the potential of recolonization from surrounding groundwater, regrowth of microbial communities surviving heat treatment, or the need for bioaugmentation for resumption of dehalogenation activity.

Effect of temperature on expression and activity of reductive dehalogenases

Temperature is a critical variable that affects enzyme stability and activity where this usually increases with increasing temperature before reaching an optimum followed by a decline in activity (Mohr and Krawiec 1980, Arcus et al. 2016). Typically the optimum temperature and thermal stability of enzymes is higher in thermophiles followed by mesophiles and psychrophiles, which is related to the corresponding 'lifestyles'. In the context of this review, the temperature behaviors of dehalogenating enzymes are of special interest. To date, several reductive dehalogenases (RDases) are described for organohalide respiration via carbon–chlorine bond cleavage (Fig. 1). PceA dechlorinates PCE and/or TCE to *cis*-DCE (Neumann et al. 1996). PCE reductive dehalogenase PteA was more recently classified in *Dhc. mccartyi* strain 11a5 and is believed to dechlorinate PCE to TCE (Jugder et al. 2016, Zhao et al. 2017, Franke et al. 2020). TCE reductive dehalogenase (TceA) dechlorinates TCE to VC via the intermediate *cis*-DCE (Major et al. 2002) and from VC to ethene (Magnuson et al. 1998, 2000, Yan et al. 2021). VC reductive dehalogenase (VcrA) is capable of dechlorinating TCE, *cis*-DCE, and VC to ethene while the BvcA dechlorinates VC to ethene. In addition, a new VC reductive dehalogenase, *cer*A, has also been reported which can dechlorinate TCE to ethene (Yang et al. 2017b).

A few of the purified CE reductive dehalogenases were characterized for their temperature optimum. Interestingly, higher temperature optima for the enzymes are reported when compared to the growth temperature optima of the respective microorganisms, e.g. PceA of *S. multivorans*, with growth optimum at 25–30°C, has its optimum at 42°C, and becomes thermolabile at 50°C (Neumann et al. 1996, Turkowsky et al. 2019). Similarly, the PceA from *Desulfitobacterium* sp. strain PCE-S has its optimum at 50°C, while the strain is optimally cultivated at 37–38°C (Miller, 1998).

A dependence of *tceA* expression on temperature in the ANAS enrichment culture was observed, with a 4-fold and 50-fold lower level of *tceA* expression (*tceA* transcripts per tceA gene) following 20 h of incubation at 22°C and 14°C, respectively, versus incubation at 30°C, while the quantity of *tceA* gene (normalized per ml) remained the same (Johnson et al. 2005). However, up-regulation of expression does not necessarily equate to dechlorination activity. For example, Fletcher et al. (2011b) showed that although *vcrA* gene transcript abundances at 35 and 40°C were one order of magnitude higher compared to at 30°C, dechlorination still stalled at VC at the higher temperatures while complete dechlorination to ethene was observed at 30°C. Their study suggests that higher gene expression does not correlate with dechlorination activity, but instead a stress response to heat (Fletcher et al. 2011b). Nonetheless, while VC dechlorination activity of culture BDI was lost at 45°C, VC dechlorination activity recovered upon returning to 24°C following incubation at 40°C for 24 d, but incubations at 40°C for longer periods resulted in the loss of dechlorination activity (Fletcher et al. 2011b). These findings exemplify how a small temperature shift can impact bacterial growth, inducing a stress response or affecting dechlorination activity.

Indirect effects of temperature on biotransformation: competing/synergetic microbial processes

Temperature can influence synergistic or competing microbial processes either by supporting, enhancing, limiting, or inhibiting dehalogenation processes or its kinetics (Fig. 3). These include production of hydrogen as electron donor and acetate as carbon source for OHRB, respectively, during fermentation, competition with the activity of methanogens and sulfate-reducing bacteria for electron donors and carbon sources, and production of cofactors (corrinoids) essential for functionality of the RDases. Generally, these processes have been reported at high temperatures (Fukui et al. 1964, Kallmeyer and Boetius 2004, Gieg et al. 2010, Verhaart et al. 2010, Piceno et al. 2014, Straub et al. 2018), however, thermophilic or thermotolerant microbial representatives may be absent in shallow aquifers. Additionally, high temperatures may result in mobilization of DOC as well as volatile contaminants (Griebler et al. 2016, Koproch et al. 2019). The increase in volatile organic compounds (VOC) concentration by mobilization from sediments may favour the faster conversion of PCE to *cis*-DCE due to better bioavailability (Ni et al. 2016); conversely, high concentrations of VOC such as PCE and TCE can be toxic to OHRB or other bacteria which support dechlorination (such as fermenters; Yang and McCarty, 2000, Huang and Becker 2011, Yoshikawa and Zhang 2020).

To our knowledge, studies which correlate temperature or temperature changes to effects which indirectly influence dechlorination are available but limited. Nonetheless, these will be described in the following subsections below.

Fermentation and production of electron donors and carbon sources for OHRB: fermentation of complex substrates, with production of hydrogen and acetate, by hyperthermophile and extremely thermophilic archaea and bacteria has been described and studied [for a review see Verhaart et al. (2010)]. Heimann et al. (2007) determined fermentation rates of lactate and pyruvate (as fast and slow hydrogen-releasing sources, respectively) at different temperatures to support anaerobic microbial dechlorination of TCE by the KB-1 consortium. Hydrogen production was observed between 4 and 40°C and the overall higher dechlorination rates of TCE to ethene could be related to the higher rates of hydrogen supply. Besides being a product of fermentation, acetate was suggested to be produced by hydrogenotrophic homoacetogenesis in lactate-amended cultures at temperatures between 4 and 30°C. No lactate fermentation was observed at temperatures above 40°C (Heimann et al. 2007). Thus, temperatures of up to 40°C generally may not hamper production of suitable electron donors and carbon sources for dehalogenation, however, it is

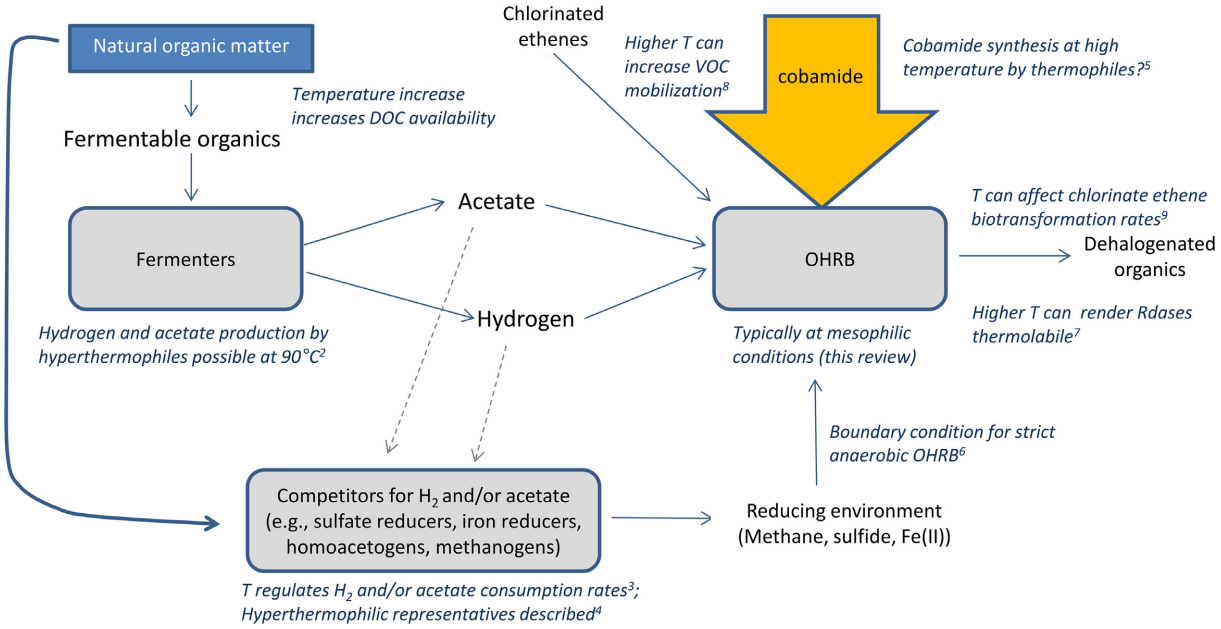


Figure 3. Summary of catabolic interactions and temperature-related bottlenecks indirectly affecting the processes; modified from Richardson (2016). (1) Friis et al. 2005, Tabuchi et al. (2010), Jesußek et al. (2013), Saito et al. (2016), and Bertolet et al. (2018), (2) Verhaart et al. (2010), (3) Conrad and Wetter (1990), Miyajima et al. (1997), Nozhevnikova et al. (2007), Tabuchi et al. (2010), Bin Hudari et al. (2020), and Metze et al. (2021), (4) Straub et al. (2018), (5) Fukui et al. (1964) and Fang et al. (2018), (6) Adrian and Löffler (2016), (7) Neumann et al. (1996) and Turkowsky et al. (2019), (8) Ni et al. (2016), and (9) Fletcher et al. (2011a, b).

questionable how and if aquifer microbial communities can retain the ability for dehalogenation at temperatures higher than 40°C and if shallow aquifers contain thermotolerant or thermophilic strains naturally.

Competition by methanogenic archaea, sulfate reducing, and iron reducing bacteria

Methanogens, sulfate reducing bacteria (SRB) and iron reducing bacteria (IRB), which may compete with OHRB (specifically CE dechlorinating bacteria) for hydrogen and acetate, have been reported to be active at psychro-, meso-, and thermophilic conditions, including extremely thermophilic archaeal representatives active at temperatures above 70°C [for reviews see e.g. Lovley et al. (2004), Gieg et al. (2010), Straub et al. (2018), Zheng et al. (2019), Lin et al. (2020)]. Nevertheless, several studies show how temperature can enhance or impede rates for methanogenesis (Sansone and Martens 1982, Schulz et al. 1997, Lettinga et al. 2001). Zhuang and Pavlosthatis (1995) described that methanogenesis and TCE dechlorination were both affected by temperature but methanogenesis was more sensitive to temperature. However, both processes showed similar temperature optima of ~ 35°C, with both members competing for the same resource pool (e.g. electron donor and carbon source; Zhuang and Pavlostathis 1995). In contrast, in a study by Fletcher et al. (2011a) involving thermal treatment showed that temperatures > 5°C impeded reductive dechlorination, but instead a majority of the reducing equivalents were utilized in methanogenesis, which was considered one of the possible cause for the incomplete dechlorination.

As with all (bio)chemical reactions, sulfate reduction rates are temperature dependent (Okabe and Characklis 1992, Elsgaard et al. 1994, Meier et al. 2005, Bonte et al. 2013, Bin Hudari et al. 2020, 2022). To our knowledge, only one study simultaneously correlated temperature and its effect on sulfate reduction and dechlorination. Guerrero-Barajas et al. (2011) observed higher rates for sulfate reduction and TCE biotransformation at 37°C compared to 70°C, however, dechlorination was thought to be cometabolic with sulfate/sulfur reduction and fermentation (Guerrero-Barajas et al. 2011).

Meanwhile, the impact of biological Fe(III) reduction on reductive dehalogenation rates is less understood (Paul et al. 2015, Di Curzio 2019, Murray et al. 2020), as well as any temperature effects on this relationship.

The role of temperature on cofactor production

Reductive dechlorination undertaken by bacteria such as *Dhc. mccartyi* strain 195, requires cobamides such as vitamin B12 for growth and as cofactor in reductive dehalogenases (Maillard et al. 2003, Butler and Kräutler 2006, He et al. 2007, Schipp et al. 2013, Fang et al. 2017). Reductive dehalogenation with vitamin B12 (cyanocobalamin) can proceed abiotically or biotically, by generating a cobalt(I) supernucleophile using a strong reductant or catalyzed corrinoid-dependent reductive dehalogenases, respectively (Kim and Carraway 2002, He et al. 2007, Heckel et al. 2018, Yan et al. 2018). OHRB are either capable of producing cobamides *de novo*, either aerobically or anaerobically or use salvage pathways such as *Dehalococcoides*, which relies on exogenous cobamides (e.g. vitamin B12) in the growth medium (Schipp et al. 2013, Balabanova et al. 2021). Variations in the lower Coα base of the cobamide can impact microbial reductive dechlorination rates and growth of *Dhc. mccartyi* strains (Yan et al. 2018), resulting in varying rates or a complete loss of dechlorination activity as well as substrate utilization (Yan et al. 2015). This can be crucial for dechlorinators such as *Dehalococcoides*, which rely on exoge-

nous cobamide, where the loss of cobamide resulting from thermal treatment could instead result in utilization of less favourable cobamides affecting the extent and rates of dechlorination. Besides, higher concentrations of vitamin B12 have been described to improve the growth of *Dehalococcoides* sp. (He et al. 2007, Fang et al. 2017), and temperature can also promote growth of reductive dehalogenase-dependent cobamide producers. One study mentioned thermophilic cobamide production (Fukui et al. 1964), however, to our knowledge, there is no information on the effects of temperature on the cobamide producers supplying cofactors beneficial to the OHRB.

Other effects of high temperature

Temperatures above 45°C significantly mobilize dissolved organic carbon (DOC) from aquifer sediments (Griebler et al. 2016), also likely providing electron donors for reductive dehalogenation. For example, Marcet et al. (2018b) incubated 14 different solid materials at temperatures 30, 60, and 90°C, and reported an increase in the release of direct electron donors (such as acetate and H_2) and fermentable volatile fatty acids (e.g. lactate and propionate), highest at 90°C followed by 60°C when compared to 30°C higher temperatures. Friis et al. (2005) observed an increase of DOC in the aqueous phase after heating contaminated aquifer material up to 100°C (Newmark and Aines 1995, Friis et al. 2005). Marnette et al. (2010) attributed higher dechlorination activity to the increased mobilization of VOC (i.e. increased desorption) available to the dechlorinators. Abiotic reductive CE dechlorination can occur in parallel with biotic reductive dechlorination under field conditions to yield acetylene as well as ethene (Dong et al. 2009). Schaefer et al. (2017) investigated abiotic reductive dechlorination between 20 and 55°C in three clayey aquifer materials, an aquitard from PCE-impacted site in Puerto Rico, PCE-impacted site in the USA, and a TCE-impacted clayey layer from New Hampshire: acetylene and ethene generation followed the Arrhenius relationship and was putatively attributed to a higher ferrous mineral content. Costanza and Pennell (2007) incubated PCE contaminated soil and groundwater at 25, 55, 75, and 95°C. Aqueous phase PCE concentrations increased with higher temperatures which is attributed to PCE mass transfer from solid to aqueous phase. TCE hydrogenolysis to *cis*-DCE occurred at higher rates at 95°C compared to 55°C (Costanza and Pennell, 2007).

Potential for active combination of thermal energy storage and bioremediation: challenges

ATES becomes increasingly relevant in urban areas due to growing energy demands in combination with the transition to sustainable, CO_2 reduced energy systems (Fleuchaus et al. 2018). On the other hand, chlorinated solvents are typically contaminants of urban industrial and residential areas and since ATES applications results in water exchange between locations during input and output the potential migration of toxic contaminants may become problematic. Besides, one should also consider other effects (e.g. chemical) on pipes in sulfidic groundwater such as pipe clogging from iron sulfide precipitates (Li et al. 2017) or pipe corrosion (Lerm et al. 2013). However, a combination of ATES with bioremediation could allow a simultaneous contamination removal while fulfilling heating and cooling demand and supply, killing two birds with one stone. Clearly, integrating thermal treatment or thermal energy storage with bioremediation requires an understanding of microbial activity and adaptation to shifting temperatures.

Combinations with LT-ATES, with temperatures up to 25°C, would likely promote biotransformation as most described strains and consortia used for bioremediation purposes are adapted to these temperatures. Nevertheless, there are demands for storing higher temperatures (HT-ATES) due to the higher energy density per volume (Drijver et al. 2012). This is particularly interesting for urban areas where heat management requires storage in smaller volumes in the subsurface. HT-ATES systems in combination with bioremediation would have several added challenges. One of the probable scenarios following the use of high temperature either in thermal treatment or HT-ATES is that these may leave the subsurface void of the native microbial community and/or the necessary microorganisms that contribute towards contaminant biotransformation (Griebler et al. 2016). As shown in Table 1 and Tables S1 and S2 (Supporting Information), most CE-dechlorinating bacteria and consortia are cultivated in mesophilic conditions, while only a limited number are capable of spore formation, allowing survival under harsh conditions. While high temperature can induce the release of DOC containing putative electron donors and carbon sources for OHRB, and thereby stimulating microbial reductive dehalogenation, these populations may not survive the high temperatures (> 50°C) of the heating phase. Thus, natural inflow of native reductive dechlorinators with the surrounding groundwater from cooler regions or bioaugmentation with laboratory-grown reductive dechlorinating cultures may be necessary. Bioaugmentation, ideally in combination with biostimulation, is a useful strategy to restore dechlorinating ability at a contaminated site (Harkness et al. 1999, Ellis et al. 2000, Lendvay et al. 2003, Friis et al. 2005, 2006, Morrill et al. 2005, Lookman et al. 2007, Fletcher et al. 2011a, Ni et al. 2014). Bioaugmentation of heat-inactivated PCE/TCE contaminated sediments with the *Dehalococcoides*-containing, PCE to ethene dechlorinating consortium OW resulted in dechlorination of at least 85% of the PCE/TCE in a microcosm study, albeit to *cis*-DCE, which was further dechlorinated to ethene after biostimulation with hydrogen gas (Fletcher et al. 2011a). However, cyclic high temperatures regimes may then similarly require a cyclic bioaugmentation regime.

Key open questions and conclusion

This review observed several gaps that would need to be addressed with respect to reductive dechlorination at higher temperatures. The first gap pertains to the lack of information on thermophilic or thermotolerant reductive dechlorinators native to the subsurface. Thus far, a majority of isolates and enrichment cultures capable of reductive dechlorination comprise of mesophiles, derived from temperate zones and cultivated at common laboratory incubator temperatures (20–30°C). There are exceptions though, such as the non-CE dechlorinating *Dsb. chlororespirans* that grows between 15 and 37°C (optimum: 37°C); while no growth was reported above 45°C, residual activity was observed to persist up to 55°C, e.g. Sanford et al. (1996), observed dechlorinating activity in resting cells for up to 11 h when incubated at 50°C (Löffler et al. 1996, Sanford et al. 1996). Therefore, in order to diversify CE-dechlorinating bacteria (OHRB in general) to include thermophiles, perhaps there should be a greater attempt to isolate thermophiles, in addition to targeted enrichments for reductive dechlorinators at naturally hot sites. While there have been efforts to explore thermophilic reductive dechlorination, these have yet to yield any cultures from such habitats. In addition, searches for sequences of reductive dehalogenases from hot environment

did not generate many outputs (if any). Second, there is a lack of comprehensive information on some isolates and strains in the presented studies, particularly with respect to the temperature range for growth, the electron donor/acceptor profile, as well as whether they are capable of spore-spore formation (Table S1, Supporting Information). Spore formation could be crucial for resumption of dechlorination following a period of higher temperatures, or is essential for putative thermophilic reductive dechlorinators being inactive but alive at temperate or cold regions. Third, for a successful combined heat storage/thermal treatment and bioremediation approaches at contaminated sites, knowledge seems to be lacking on the direct effects of temperature perturbations on the microbial community, especially the dechlorination potential and recovery of dechlorination capacity. Fourth, unlike the extensive studies done to characterize isolates and enrichment cultures, much fewer studies pertaining to field site applications are available to our knowledge; perhaps, these studies were not made public for commercial or legal reasons. The fifth gap pertains to the very limited information available about the indirect effects of temperature on dechlorination, e.g. effects on syntrophic partner organisms or competitors of electron donors. The role of temperature on DOC and associated release of electron donors or toxicity from increased VOC mobilization for reductive dechlorination should also be investigated in greater detail. The penultimate gap, seems to be a lack of knowledge on the consortia applied for dechlorination for bioaugmentation [for e.g. KB-1 see Table S2 (Supporting Information)], and whether they are resilient to temperature shifts. Lastly, based on our literature search (Table S2, Supporting Information), very little information documenting dechlorination potential in ATES is available, with only a few studies related to thermal treatment.

In summary, there has been significant progress in the isolation and characterization of OHRB, as well as consortia for the purpose of bioremediation. This review highlights the gap when bioremediation is coupled with thermal energy storage and the need for resilient, active thermophilic OHRB sustaining biodegradation reactions at high temperatures. However, it is also important to note that other parameters can also directly and indirectly affect dehalogenation. Above all, since bioremediation is also site-specific and dependent on the type of contaminants and the native microbial community present, a comprehensive site survey is firstly necessary prior to establishment of bioremediation coupled to thermal energy storage.

Supplementary data

Supplementary data are available at *FEMSEC* online.

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Chapter 6.

Temperature-associated effects on methanogenesis and microbial reductive dechlorination of trichloroethene in contaminated aquifer sediments

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Temperature-associated effects on methanogenesis and microbial reductive dechlorination of trichloroethene in contaminated aquifer sediments

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Background: Aquifer thermal energy storage (ATES) is a subsurface technology for urban heating and cooling. However, ATES systems may intersect with legacy groundwater contaminants from past anthropogenic activities. Chlorinated ethenes, particularly tetrachloroethene (PCE) and trichloroethene (TCE), are common pollutants that can undergo microbial reductive dechlorination to *cis*-dichloroethene (*cis*-DCE), vinyl chloride (VC), and ultimately ethene. Since microbial activity is temperature dependent, heat storage in ATES systems may influence dechlorination efficiency.

Methods: The study assessed the effect of temperature on microbial reductive dechlorination and community composition using sediment from a contaminated aquifer in Ferrara, Italy, where VC accumulation is of concern. Laboratory microcosms were amended with TCE and lactate, incubated at 10–60°C, and monitored for 105 days.

Results: Complete dechlorination to ethene occurred at 10–20°C and was linked to *Dehalogenimonas* spp. *cis*-DCE and VC accumulated at 30°C and 40°C, respectively, while no dechlorination activity was observed at 50°C and 60°C, suggesting temperature-related inhibition. Methanogenesis occurred between 10 and 40°C and was associated with *Methanosarcina*, *Methanothrix* (mainly in non-TCE-amended controls), and *Methanomicrobia* (10–30°C). Methanogenic activity was absent above 40°C and delayed at 10°C.

Conclusion: These results suggest that microbial dechlorination of chlorinated ethenes is impaired at temperatures exceeding 40°C. Therefore, integrating low- or medium-temperature (<40°C) ATES with enhanced natural attenuation may offer a viable strategy for simultaneous energy storage and bioremediation in chlorinated solvent-contaminated aquifers.

KEYWORDS

ATES, reductive dechlorination, methanogenesis, temperature, chlorinated ethenes, bioremediation

Introduction

Aquifer thermal energy storage (ATES) is a subsurface technology for urban heating and cooling, offering a promising solution to reduce dependence on fossil fuels (Mathiesen, 2019), especially in densely populated areas with high energy demands (Elsland et al., 2017; Menberg, 2014). However, many urban aquifers are contaminated due to anthropogenic

activities, including the release of chlorinated ethenes (CEs) through leakages and improper disposal practices (Bishop et al., 1993; Rittmann et al., 2000; Ruden, 2006).

Chlorinated ethenes (CEs), i.e. tetrachloroethene (perchloroethene, PCE), trichloroethene (TCE), the dichloroethene isomers (DCEs), and vinyl chloride (VC), are widely used in commercial and industrial applications, including as solvents, cleaning agents and plastics manufacturing. Due to their environmental persistence, toxicity, and frequent occurrence in urban groundwater systems, these compounds are classified as priority pollutants, subject to strict regulatory control to safeguard surface and drinking water quality (Kalnins et al., 2019; Lepom et al., 2009; Rivett et al., 2012; Squillace et al., 2004).

Under anoxic conditions, PCE and TCE can undergo microbial reductive dechlorination to *cis*-DCE and VC, and ultimately to the non-toxic end product ethene (Adrian and Löffler, 2016). Ensuring complete conversion to ethene is critical for effective bioremediation (Figure 1) (Huang et al., 2014; National Research Council, 2000).

In the subsurface, microbial dechlorination activity is influenced by environmental conditions, notably temperature (Badin et al., 2016; Wiedemeier et al., 1998). Temperature shifts can alter the composition of native microbial consortia composition and affect their reductive dechlorination ability (Beyer et al., 2016; Garcia et al., 2018; Yamazaki et al., 2022). Microbial reductive dehalogenation (RDH) to ethene has been observed up to 30°C; above this, this process often becomes incomplete—resulting in accumulation of DCE or VC—or is entirely inhibited (for a review, see Bin Hudari et al., 2022a). Temperature also influences microbial competition for electron donors and carbon sources. For instance, hydrogenotrophic methanogenesis may compete with dehalogenators and affect overall dechlorination potential (Smatlak et al., 1996; Wei et al., 2016). Methanogenesis can occur concurrently and typically spans a broader and higher temperature range (Jones et al., 1987; Prondzinsky et al., 2023).

Thus, moderately increasing subsurface temperatures (e.g., up to ~30°C) may support both ATES and enhanced contaminant bioremediation, while avoiding the inhibitory effects observed at higher temperatures (Badin et al., 2016; Bonte et al., 2013; Delille et al., 2004; Ni et al., 2018, 2015, 2016). However, for reasons of energy efficiency, there is growing interest in increasing groundwater reinjection temperatures in low-temperature (LT)-ATES (typically ≤25°C) to >50°C for high-temperature (HT)-ATES applications. HT-ATES systems offer greater sustainability for heating and cooling due to their higher energy storage capacity (Daniilidis et al., 2022; Drijver et al., 2012; Kallesøe and Vangkilde-Pedersen, 2019). This creates a trade-off between thermal energy optimization and maintaining favorable conditions for microbial contaminant degradation.

Despite its importance, few systematic studies have investigated the effects of temperature on microbial reductive dehalogenation, and even fewer that address shifts in the associated microbial communities. In particular, data on how elevated temperatures affect bioremediation *in situ* remain limited (for a comprehensive review, please see Bin Hudari et al., 2022a).

This study aimed to investigate the impact of increasing temperature on microbial reductive dehalogenation activity and microbial community composition using contaminated sediments in a controlled laboratory setting across a broad temperature range.

Materials and methods

Chemicals

All chemicals used in the experiments were purchased from Merck (Darmstadt, Germany), AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma-Aldrich (Deisenhofen, Germany) and were of analytical grade at the highest purity.

Laboratory microcosm preparation

Sediment samples were collected from cores drilled at a chlorinated ethenes-contaminated site in northern Italy, near a former disposal area where chlorinated pitches from chloromethane production were illegally dumped between the 1950s and 1970s (Ghezzi et al., 2021; Nijenhuis et al., 2013). Field observations have indicated ongoing dechlorination of PCE and TCE at this site—where natural groundwater temperatures range from 16 to 20°C—with vinyl chloride (VC) often accumulating as the primary or sole detectable intermediate (Filippini et al., 2016). For the microcosm study, TCE was selected as the model compound and its reductive dehalogenation was investigated at six different temperatures (10, 20, 30, 40, 50, and 60°C). Product formation was monitored over 105 days, after which the bacterial and archaeal community structures were analyzed.

The sediment samples used in this study were obtained from a well-characterized hydrogeological setting comprising vertically stacked sandy aquifers and clayey aquitards (Filippini et al., 2016, 2020). PCE and TCE, originally contained in DNAPL (dense

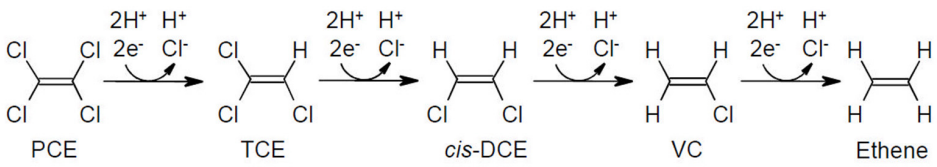


FIGURE 1
Reductive dechlorination pathway of PCE to ethene via TCE, *cis*-DCE, and VC. Each step involves microbial replacement of a chlorine atom with hydrogen under anoxic conditions.

non-aqueous phase liquids) wastes, have migrated downward into the subsurface, resulting in chlorinated ethene contamination detected in groundwater and sediments to depths of up to 50 meters below ground surface (bgs) reaching hundreds of mg L^{-1}. Sediments for the microcosms were collected from two core sections: one aquifer layer between 15 and 25 m bgs (corresponding to the "Upper A1" aquifer) and one aquitard layer between 25 and 30 m bgs (part of the "Lower Q1" facies). Sediments were obtained from cores collected during previous site investigations described by Nijenhuis et al. (2013) and Filippini et al. (2016); details of the drilling and sampling procedures can be found in those studies. These cores were recovered during a detailed site investigation at the Caretti site in 2013 (boreholes MC1-2, MC3, and MC4-5), which also included stratigraphic reconstruction using direct-push drillings, multilevel monitoring wells, and piezocone penetration testing (Filippini et al., 2016; Nijenhuis et al., 2013).

Microcosms were set up under anoxic conditions in a glove box (COY Laboratory Products Inc., Michigan, USA), maintained with an N_2/H_2 gas mixture (95:5%). This atmosphere ensured strict anoxic conditions; while H_2 can act as an electron donor, lactate (3 mM) was provided as the primary electron donor and carbon source to support microbial reductive dechlorination. The intent was to ensure that electron donor availability was sufficient, though lactate was not added in large excess. All materials—including serum bottles, septa, and crimps—were autoclaved, dried, and placed inside the glove box several hours before use to prevent oxygen intrusion. Sediments were consolidated and homogenized inside the glove box. A total of 48 serum bottles (120 mL) were prepared, each comprising 20 g of sediment and 50 mL of mineral salts medium (composition in Supplementary Table S1) (Zinder, 1998). Bottles were sealed with Teflon coated rubber septa and aluminum crimps. Bottles were incubated lying on their sides to ensure that the liquid medium covered the septum, minimizing the risk of air intrusion through the septum after perforation.

Eight bottles were assigned to each of six different temperatures (10°C, 20°C, 30°C, 40°C, 50°C, and 60°C): five active replicates (A, B, C, F, G), two sterile controls (D and E), and one anaerobic, non-amended control (ANA) to monitor background activity such as methanogenesis or reductive dechlorination. Sterile replicates were autoclaved at 121°C for 40 min on three consecutive days (see Supplementary Table S2). All bottles were pre-acclimatized at the respective temperatures for at least 48 h before initiating the experiment. The experiment began with the addition of lactate (3 mM) and ∼5 µL of neat TCE, resulting in an estimated starting concentration of 100 µmol L^{-1}. ANA controls received no TCE. After amendment, bottles were equilibrated for 3 h at their target temperature prior to sampling. For sampling, 0.5 mL of the headspace was taken from each bottle using a Hamilton gas syringe, with gas transferred to a helium-flushed 10 mL gas chromatography (GC) headspace vial. Following TCE depletion, additional doses (100 µmol L^{-1}) were added. The 20 and 30°C replicates received a total of four doses of TCE (0.4 mmol L^{-1} total).

Chemical analysis

Samples were analyzed via gas chromatography coupled with a flame ionization detector (GC-FID; Varian Chrompack CP-3800), with a GS-Q column (J&W Scientific, Waldbronn, Germany) and injected via a headspace autosampler HP 7694 (Hewlett Packard, Palo Alto, USA). The chromatographic separation program was adapted from Nijenhuis et al. (2007) and initially set to 100°C (held for 1 min), followed by a temperature ramp of 50°C min^{-1} to 225°C (held for 6 min). To improve separation of overlapping peaks—particularly as ethene and methane concentrations increased during incubation—the program was further modified after 63 days to start at 80°C (1 min), with the same ramp to 225°C (6 min hold). Data was analyzed by the Varian STAR software for the respective target compounds to obtain the area counts and concentrations were calculated using independent calibration curves for TCE, *cis*-DCE, VC, ethene, ethane, and methane (not shown).

Statistical analyses were performed in R (version 2024.12.1) using pairwise Wilcoxon rank-sum tests with Benjamini–Hochberg correction for multiple comparisons, based on the highest accumulated concentrations of ethene and methane across temperature treatments. Significant differences between treatments are indicated in the boxplots.

Microbial community analysis

The microcosms were sacrificed after 397 days of incubation. While the relevant results shown and discussed in this manuscript reflect observations up to day 105, active microcosms were maintained until day 397 through periodic amendment with TCE as the electron acceptor. TCE was re-supplied only when measurements indicated it had been fully consumed. Two to four mL of sediment slurry were collected from 36 of the sample bottles, except for the 12 sterile controls, into 2 mL Eppendorf tubes (see Supplementary Table S3). The slurry was centrifuged at 13 000 rpm for 2 min, the supernatant was discarded, and the pellet was stored at −20°C until extraction. At each temperature, one non-amended replicate (i.e., without TCE) was included to serve as background control, allowing qualitative assessment of microbial community composition in the absence of TCE. These background samples were not included in statistical comparisons but supported interpretation of temperature-related shifts in microbial profiles. DNA extraction was carried out with the DNeasy Powersoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with minor modifications.

For each replicate, sediment slurries from a single microcosm bottle were distributed into multiple Eppendorf tubes and processed in parallel. Prior to the elution step, lysates from the same replicate were sequentially loaded onto a single spin column during centrifugation, allowing DNA to be pooled and concentrated. The DNA was eluted in a final volume of ∼20–30 µL. Specifically, the elution step was performed by first adding 20 µL of elution buffer onto the column membrane, letting it stand for 1 min before centrifugation. This step was repeated using the collected eluent (15 µL) combined with an additional 15 µL of buffer to maximize DNA recovery. DNA concentration was measured with the Qubit HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, USA) on the Qubit 3.0 Fluorometer (Life Technologies, Malaysia). MiSeq sequencing procedures were similar to previous studies prescribed elsewhere (Bin Hudari et al., 2020, 2022b) using the Klindworth primer pair (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21).

Briefly, sequencing libraries were assembled with Illumina MiSeq Reagent Kit v3 (2 x 300 bp) following protocols recommended by the manufacturer on 16S Metagenomic Sequencing Library Preparations (Illumina, 2013). Sequencing was done on the Illumina Miseq platform at the former Department of Environmental Microbiology (currently, Applied Microbial Ecology) of the Helmholtz Centre for Environmental Research—UFZ. Sequences were analyzed on a QIIME 2 v2019.1 platform using a pipeline as described previously (Bolyen et al., 2019). This pipeline removes primer sequences and adapters from the de-multiplexed sequences, then trimming and denoising them to remove low quality reads and chimeras, before merging. Amplicon sequence variants (ASVs) were then assigned to the bacterial DNA using the Silva132 database (Quast et al., 2013; Yilmaz et al., 2014). Sequences were deposited at the European Nucleotide Archive (ENA) under the primary accession number PRJEB73360 (https://www.ebi.ac.uk/ena/browser/view/PRJEB73360).

Results

Reductive dechlorination and methanogenesis at different temperatures

TCE dechlorination product formation was monitored for up to 105 days to highlight treatment-specific differences in activity. Product profiles varied with temperature: ethene was detected at 10–30°C, with ethane appearing at 20°C and 30°C, indicating more complete dechlorination at these temperatures. VC was observed only at 30°C, while *cis*-DCE accumulated at 30°C and 40°C, suggesting incomplete dechlorination at higher temperatures. No dechlorination products were detected at 50°C and 60°C.

Figure 2 presents representative data from 20, 30, and 40°C to illustrate the temperature-dependent transition from complete to incomplete dechlorination. These temperatures were selected to highlight the effects of thermal elevation on dechlorination and methanogenesis. The 10°C condition, which reflects the native groundwater temperature and exhibited complete dechlorination, is shown in Supplementary Figures S1A–C due to space limitations. Additional replicate data for all conditions, including full triplicate datasets at 10°C and 60°C, are provided in Supplementary Figures S1–S3.

Methanogenesis and reductive dechlorination were temperature dependent and inhibited at higher temperatures (≥50°C). Dechlorination occured at 10–40°C, though the extent decreased at 30 and 40°C. Complete TCE dechlorination to ethene was observed at 10°C (Supplementary Figures S1A–C), 20°C (Figure 2A; Supplementary Figures S1E–G), and 30°C (Figure 2C; Supplementary Figures S2A–C), with ethene concentrations increasing over time. No activity was observed in sterile controls (data not shown).

In setups at 10°C, following a lag phase, ethene accumulated after 50 days without detectable *cis*-DCE or VC accumulation (Supplementary Figures S1A–C). Methanogenesis was not significant during the early phase (<0.1 mmol L^{-1} of methane; Supplementary Figure S1D) but was observed after 130 d of incubation, including in the non-amended controls (data not shown). At 20°C and 30°C, ethene was observed after 10–20 days and increased before declining after 80 days, accompanied by rising ethane concentrations (Figures 2A, C; Supplementary Figures S1E–G, S2A–C). Methane accumulation in the TCE-amended replicates ranged between 11 and 18 mmol L^{-1} at 20°C (Figure 2B, Supplementary Figure S1H) and 16–31 mmol L^{-1} at 30°C (Figure 2D; Supplementary Figure S2D).

At 30°C, VC began to accumulate after 63 days, reaching stable levels (~0.4 mmol L^{-1}), while ethene remained the primary product until 42 days, after which both ethene and ethane were detected (Supplementary Figures S2A–C). At 40°C, dechlorination stalled at *cis*-DCE by day 105 (Figure 2E; Supplementary Figures S2E–G), and no further transformation occurred even after extended incubations (397 days; data not shown). Methane accumulation in 40°C TCE-amended replicates was comparable to that at 20°C, ranging between 11 and 25 mmol L^{-1} (Figure 2F; Supplementary Figure S2H). Notably, methane production occurred to a similar extent in the non-amended controls, indicating that it was not directly coupled to TCE amendment.

In general, the total measured concentrations of chlorinated ethenes in microcosms incubated at 10–40°C exceeded the initial TCE input. This discrepancy is likely due to residual CEs in the sediment and potential sampling artifacts, such as increased partial pressures at higher temperatures or elevated headspace pressure resulting from methane production.

To summarize treatment effects across all conditions, ethene and methane concentrations were compared using boxplots and Wilcoxon rank-sum tests (Figure 3). Ethene production peaked at 20°C and 30°C, with statistically significant differences ($p \leq 0.05$) compared to all other treatments. Methane showed a similar trend, with the highest concentrations at 20–30°C, moderate levels at 40°C, and little or no production at 10°C, 50°C, or 60°C. These results confirm a temperature optimum between 20 and 30°C for both reductive dechlorination and methanogenesis, while no significant product formation was observed at ≥50°C.

The microcosms were monitored until day 397 to evaluate possible delayed activity in the 50°C and 60°C treatments. However, no dechlorination or methanogenesis was observed (Supplementary Figures S3A–H). A slight decrease in TCE concentration was noted, but no intermediate or end products accumulated, and these trends mirrored those of sterile controls. Meanwhile, active microcosms (e.g., 20°C and 30°C) were maintained with occasional TCE supplementation until the end of the experiment and were later sacrificed for microbial community analysis. While results up to 105 days and microbial community profiles are shown, data from the extended incubation period (105–397 days) are not shown.

Microbial community analysis

A total of 36 samples—comprising five TCE-amended biological replicates and one non-amended active control per temperature—were sequenced using 16S rRNA gene primers

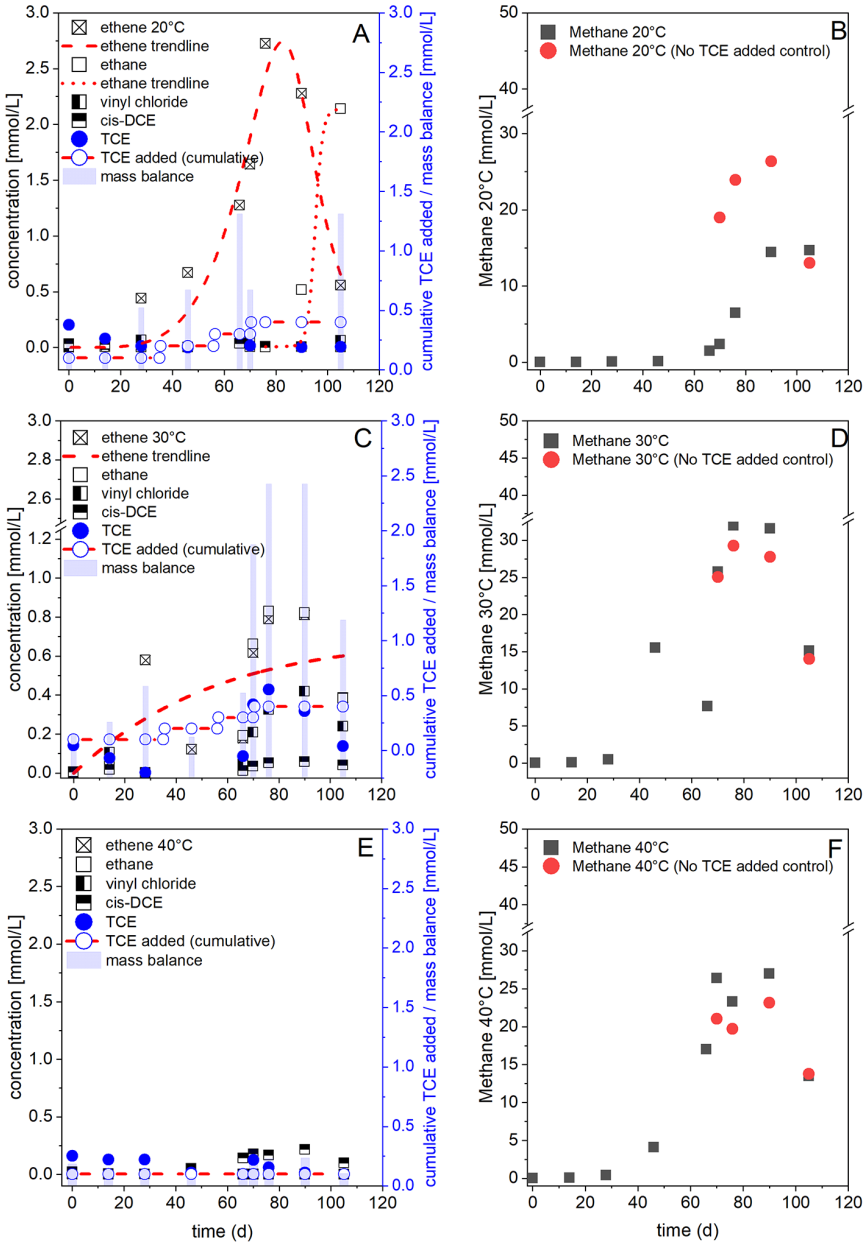


FIGURE 2
Representative profiles of trichloroethene (TCE) dechlorination and methanogenesis in microcosms incubated at 20°C **(A, B)**, 30°C **(C, D)**, and 40°C **(E, F)**. **(A, C, E)** show concentrations of TCE, its dechlorination products (*cis*-DCE, vinyl chloride, ethene, ethane), and the cumulative TCE mass balance over 105 days. "Mass balance" represents the total molar sum of TCE and its dechlorination products. **(B, D, F)** show methane production in TCE-amended microcosms (black) and in the corresponding non-amended anaerobic controls (red). These replicates are representative; additional replicate data are provided in Supplementary Figures S1–S3.

targeting bacteria. This approach generated an average of 72,867 reads per sample (±16,160), with read counts ranging from 42,205 to 104,396 reads. To assess methanogenic community composition, an additional eight samples (one replicate with and one without TCE amendment per temperature, for 10–40°C) were sequenced with the methanogen specific *mcrA*-targeted primers, targeting the methyl coenzyme M reductase gene (Supplementary Table S3). This *mcr*A sequencing generated an average of 75,749 reads per sample (±20,636), with read counts between 51,018 and 113,905. While the *mcrA*-based dataset provided valuable insight into methanogen diversity, we acknowledge that the use of single replicates precludes statistical analysis. Community composition profiles from one representative replicate per active temperature treatment (10–40°C) are shown in Figures 4, 5. Additional replicate data are provided in Supplementary Figures S4, S5 at both family and genus levels.

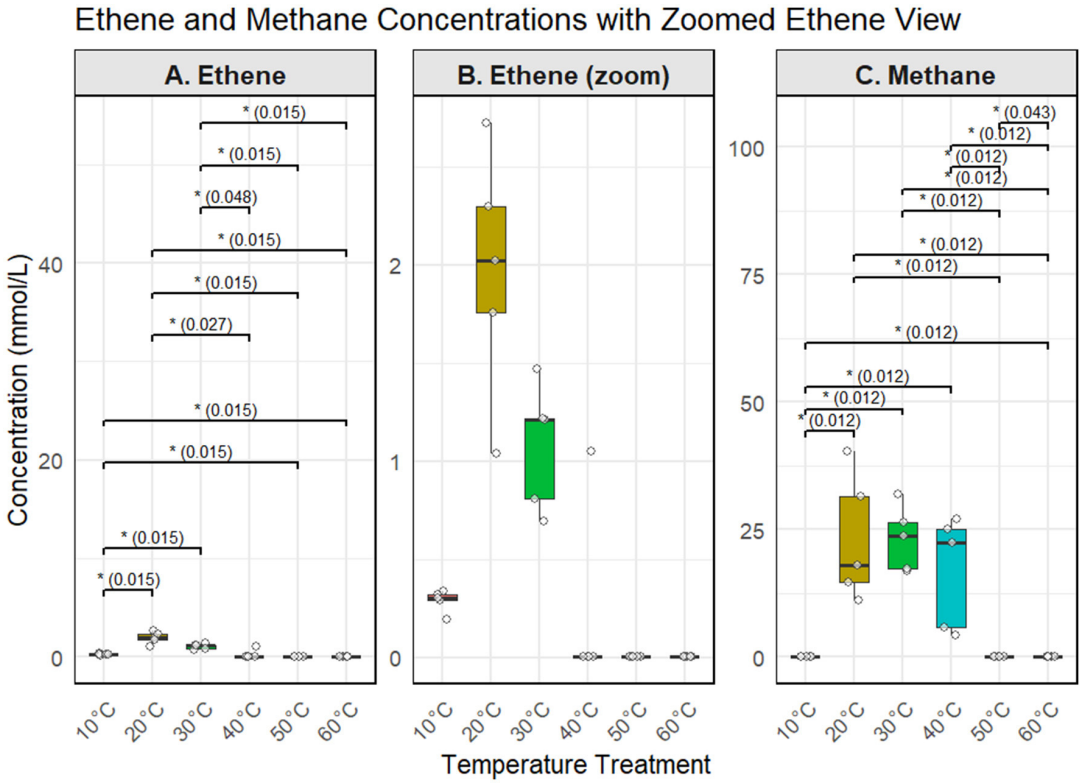


FIGURE 3
Maximum observed concentrations of ethene and methane in microcosms across temperature treatments. **(A)** Ethene; **(B)** Zoomed view of ethene (≤3 mmol L^{-1}); **(C)** Methane. Boxplots represent the highest concentration measured in each replicate. Individual replicate values are overlaid as jittered points. Statistically significant differences between treatments (Wilcoxon test, Benjamini–Hochberg corrected) are indicated by asterisks with corresponding adjusted *p*-values.

Phylotypes observed in 10°C and 20°C replicates (with and without TCE) were relatively similar in composition (Figure 4 and Supplementary Figures S4, S5). *Dehalogenimonas* was consistently present at 17–27% relative abundance in all four microcosms (Figure 4). *Acetobacterium* was more abundant in the 10°C TCE-amended (28%) and 20°C non-amended (ANA) replicates (25%) but was less dominant (4–5%) in the corresponding reciprocal treatments. In contrast, phylotypes such as *Acidaminobacter* and *Cryptanaerobacter* showed higher relative abundances specifically in the TCE-amended 20°C microcosms. Other phylotypes detected at lower abundance (3–9%) included uncultured *Spirochaetaceae* members and *Geobacter*.

At 30°C, *Cryptanaerobacter* reached 8% in the 30°C TCE-amended replicate shown in Figure 4 and was also detected in the other replicates (Supplementary Figure S5). *Dehalogenimonas* remained present, albeit at lower levels (0.2–1.2%), while uncultured *Spirochaetaceae* (17%), *Acidaminobacter* (8%), *Desulfovibrio* (3%), and *Desulfobulbus* (2%) were also identified. At 40°C, the TCE amended replicate was dominated by *Sporomusa* (26%), uncultured *Spirochaetaceae* (12%) and *Desulfobulbus* (7%) while *Dehalogenimonas* was nearly absent (0.4%), indicating a potential temperature threshold for its activity or survival.

Methanogen community composition (10–40°C) is shown at the genus level in Figure 5. One TCE-amendment and one non-amended replicate was analyzed per temperature. At 10°C—despite the absence of methane production during the first 105 days (Supplementary Figure S1D), methane was detected after 130 days (data not shown), justifying the inclusion of this sample in the analysis. At 10°C, *Methanosarcina* was the dominant genus in both amended (40%) and non-amended (45%) microcosms (Figure 5). The amended setup also contained *Methanomicrobia* (32%) and *Methanoregula* (13%), whereas the non-amended replicate had higher *Methanoregula* (26%) and *Methanothrix* (15%).

At 20°C, unknown *Methanomicrobi*a members were abundant in both the amended (51%) and non-amended (39%) setups, followed by *Methanosarcina* (25% and 21%, respectively). *Methanolinea* (11% and 7%) and *Methanothrix* (2% and 19%) were also present. At 30°C, *Methanosarcina* (47% and 29%) and an unknown member of *Methanomicrobia* (33% and 14%) dominated both treatments. *Methanoculleus* was more abundant in the TCE-amended replicate (13%), while *Methanothrix* (44%) was predominant in the non-amended replicate.

At 40°C, *Methanosarcina* abundance decreased in the TCE-amended setup (12%), while *Methanoregula* and *Methanolinea* —previously detected at lower temperatures—were absent. In contrast, *Methanocella* (39%) and *Methanoculleus* (36%) became more prominent, suggesting a shift toward thermotolerant methanogens. In the corresponding non-amended replicate,

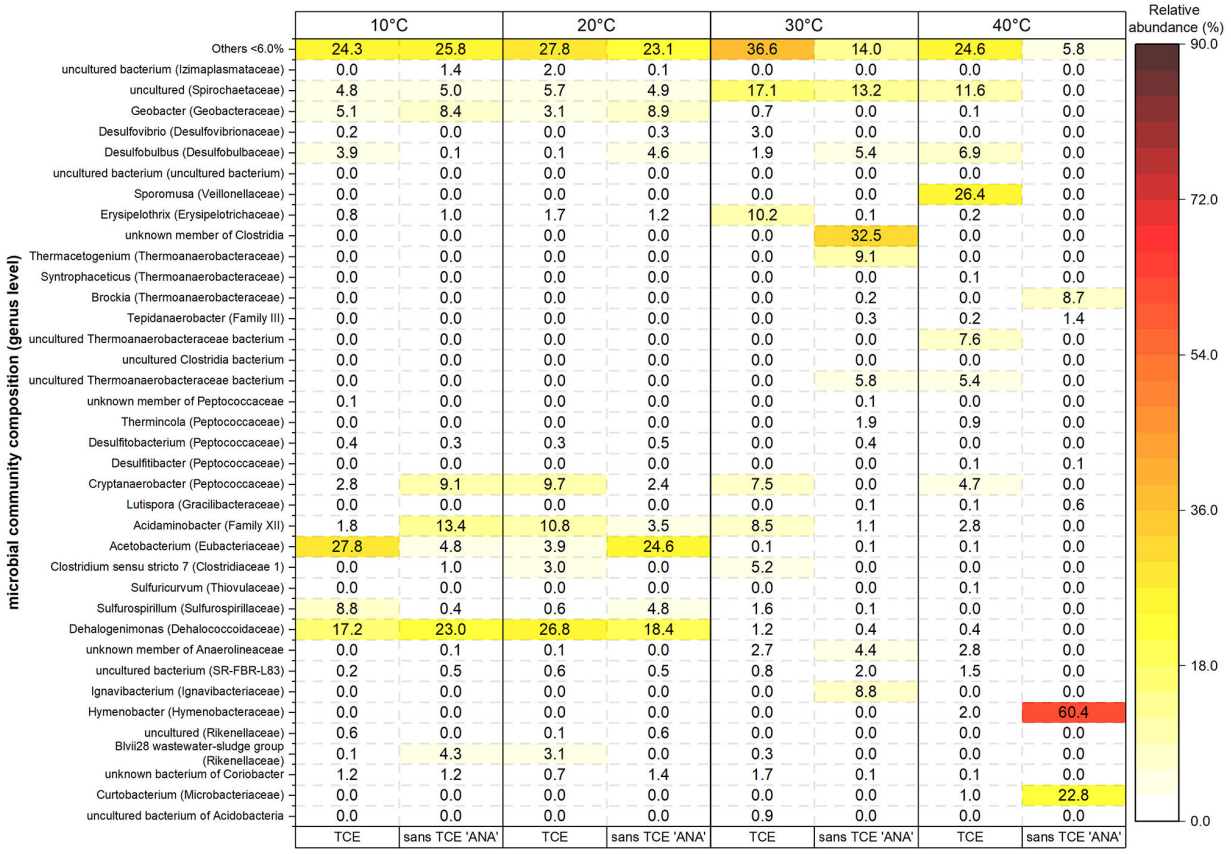


FIGURE 4
Relative abundance (%) of microbial genera (≥6% in at least one condition) across temperature treatments (10–40°C) under anaerobic conditions, with and without trichloroethene (TCE). Warmer heatmap colors indicate higher abundance. Conditions are labeled as "TCE" (with TCE) and "sans TCE 'ANA"' (anaerobic control). Genera below 6% are grouped as "Others <6.0%."

Methanosarcina (18%) and Methanothrix (48%) remained abundant, consistent with patterns observed in non-amended replicates at lower temperatures. Notably, *Methanobacterium* reached 32% in the 40°C non-amended replicate, indicating a possible temperature- and treatment-specific niche for this genus.

Discussion

Temperature significantly influenced both microbial community composition and the extent of TCE dechlorination, leading to *cis*-DCE and VC accumulation at 40°C and 30°C, respectively. Dechlorination rates were highest at 20–30°C, while temperatures >40°C were inhibitory to both reductive dechlorination and methanogenesis. Complete dechlorination to ethene occurred only in microcosms incubated between 10 and 30°C, confirming the temperature sensitivity of this process, as shown in earlier studies (Friis et al., 2007b; Heimann et al., 2007). A 10°C temperature increment (e.g., from 30 to 40°C) affected the transformation sequence, leading to VC accumulation at 30°C and *cis*-DCE at 40°C after 60 days. At higher temperatures (50–60°C), neither dechlorination nor methanogenesis occurred, consistent with previous reports (Friis et al., 2007b; Zhuang and Pavlostathis, 1995). This likely reflects the inability of key dechlorinating microbes to remain viable or competitive at these elevated temperatures (Fletcher et al., 2011; Magnuson et al., 1998).

Despite a lag phase at 10°C, complete TCE dechlorination to ethene was eventually observed, similar to findings at low temperatures—including 4, 10, and 15°C—in other studies (De Bruin et al., 1992; Heimann et al., 2007). In our study, ethene formation began after ~50 days, indicating delayed microbial activity, likely due to slower metabolism or growth rates at suboptimal temperatures.

Community analysis suggests that *Dehalogenimonas* (*Dhgm.*) is a likely candidate for TCE dechlorination to ethene at 10–20°C. For example, isolate *Dehalogenimonas etheniformans* has been reported to dechlorinate TCE, DCE, and VC to ethene at 15–34°C (Chen et al., 2022; Cui et al., 2023). Here, *Dehalogenimonas* reached 17–27 % relative abundance at 10–20°C in line with complete dechlorination (Figure 2). Conversely, its low abundance (~1%) at ≥30°C corresponded to partial TCE dechlorination, suggesting thermal inhibition. Although some *Dehalogenimonas* phylotypes were present at 60°C, no dechlorination occurred, reinforcing their temperature sensitivity. At 40°C, *Dehalogenimonas* was absent; instead, phylotypes such as *Peptococcaceae* (e.g., *Desulfitobacterium*) or *Geobacter*, known

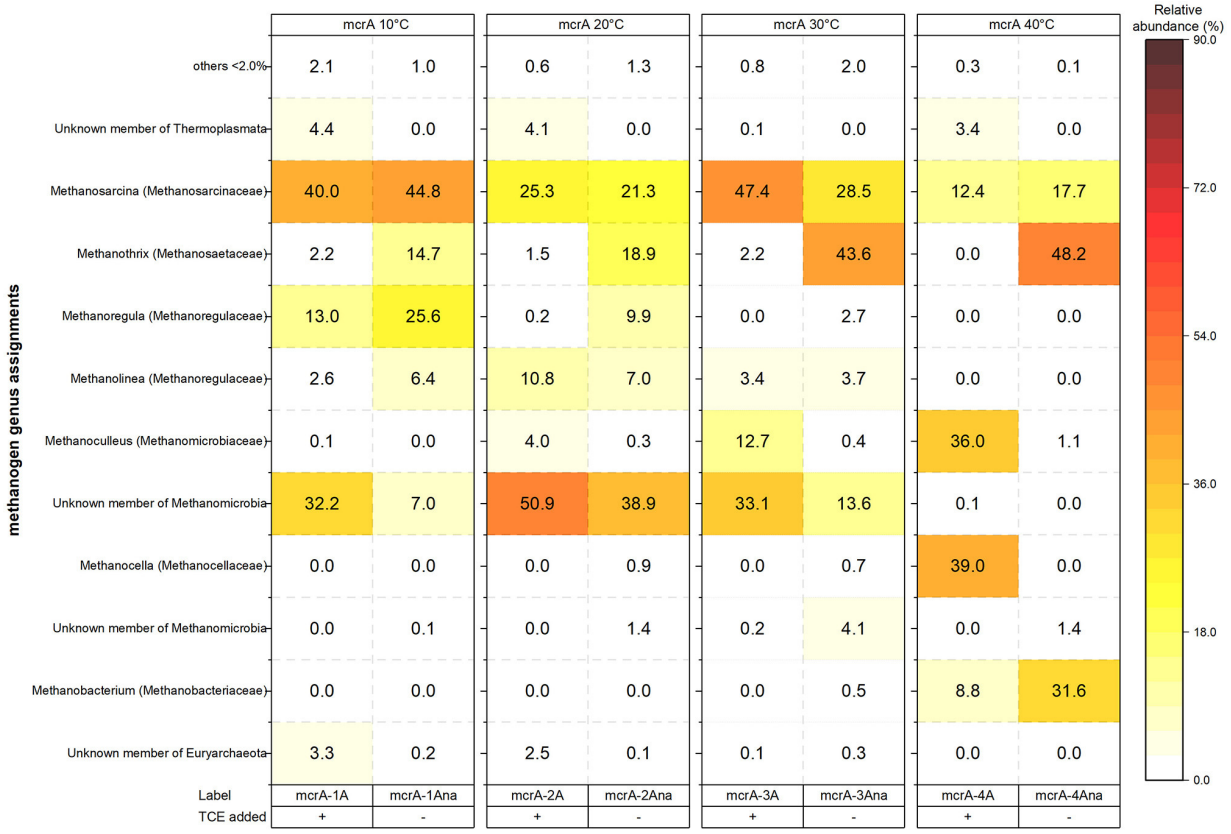


FIGURE 5
Methanogenic community composition (genus level) across temperature treatments (10–40°C) in TCE-amended (+) and unamended (–) anaerobic microcosms. Relative abundances (%) are based on *mcrA* gene sequencing. Genera ≥2% in at least one condition are shown; others are grouped as "<2%." Color intensity reflects relative abundance.

to reduce PCE to *cis*-DCE, may have contributed to TCE transformation (Röling, 2014; Villemur et al., 2006).

Although lactate degradation was not directly measured, the occurrence of reductive dechlorination and methanogenesis at 10–40°C suggests that fermentation likely occurred, providing the electron donors such as acetate and hydrogen. Lactate, added as both an electron donor and carbon source, is typically converted by a metabolically diverse microbial consortium into substrates used by organohalide-respiring bacteria (e.g., *Dehalogenimonas*) (summarized in Supplementary Figure S6) (McInerney et al., 2009; Schink and Stams, 2006; Stams and Plugge, 2009). Acetate is essential for biomass synthesis in dehalogenators (He et al., 2002; Robles et al., 2021; Rosell et al., 2019), while acetate and hydrogen can also be consumed by hydrogenotrophic and acetotrophic methanogens (Conrad, 2020; Jones et al., 1987).

Community composition analysis revealed temperature- and treatment-dependent trends among key microbial taxa. *Acetobacterium* showed notably contrasting patterns: it was abundant in the 10°C TCE-amended (27.8%) and 20°C non-amended (24.6%) replicates, but nearly absent (<5%) in the reciprocal conditions. This suggests a potential interaction between TCE exposure and temperature on *Acetobacterium* abundance or activity. Given its roles in lactate fermentation, hydrogen production, and corrinoid biosynthesis, *Acetobacterium* may have supported syntrophic partners such as dehalogenators under selective conditions (Puentes Jacome et al., 2019; Wen et al., 2015).

At 40°C, *Sporomusa* was more abundant (26.4%) in the TCE-amended setup, possibly serving as a temperature-adapted fermenter. In contrast, *Dehalogenimonas*, a key organohalide-respiring bacterium, was prominent at 10–30°C but declined at 40°C. Sulfate-reducing genera such as *Desulfovibrio* and *Desulfobulbus*, capable of incomplete lactate oxidation, were detected at 10–30°C and may have contributed to hydrogen cycling in both amended and non-amended systems.

Hymenobacter dominated one of the 40°C non-amended replicates (>60%), despite being primarily aerobic or facultatively anaerobic and not typically associated with anaerobic degradation. Its prevalence may reflect reduced microbial competition, thermal stress tolerance, or sequencing variability.

Spirochaetaceae were detected across treatments, with higher abundance at 30°C and 40°C. Species such as *Rectinema cohabitans* are known necromass feeders or acetate producers (Dollhopf et al., 2001; Dong et al., 2018; Koelschbach et al., 2017; Ritalahti et al., 2012). Some *Spirochaetes* can also oxidize acetate to produce hydrogen and carbon dioxide (Cheng et al., 2022; Si et al., 2016; Wang et al., 2019; Yi et al., 2020), and may be able to utilize lactate anaerobically (Troshina et al., 2015), suggesting a role in carbon and electron donor cycling.

These observations underscore the interplay between temperature and TCE in shaping microbial community structure and function, with implications for fermentation, methanogenesis, and reductive dechlorination dynamics.

The temperature range for ethene-to-ethane formation was narrower (20–30°C) than that observed for complete reductive dechlorination (10–30°C) or methanogenesis (20–40°C), suggesting that ethane formation may be coupled to other biological processes (Belay and Daniels, 1987; Fullerton et al., 2013; Koene-Cottaar and Schraa, 1998; Xie et al., 2013). For example, in a methanogenic consortia, members of the *Methanomicrobiales* have been postulated to reduce ethene to ethane, and this conversion was shown to be inhibited by the methanogenesis inhibitor BES (Koene-Cottaar and Schraa, 1998; Xie et al., 2013). In our study, ethene-to-ethane conversion coincided with complete dechlorination and methanogenesis at 20–30°C, supporting ethanogenesis within this temperature range.

Methanogenesis occurred at 20–40°C within 95 days and at 10°C after 130 days, but not at 50–60°C, even after extended incubation (up to 397 days). This indicates that the indigenous microbial community is adapted to mesophilic conditions (15°C to 40°C). Notably, methane production occurred in both TCE-amended and non-amended microcosms, indicating that methanogenesis was independent of TCE presence and likely fueled by fermentation-derived substrates such as hydrogen and acetate. Although methanogenesis can occur between −2.5 and 122°C in other systems (Jones et al., 1987; Mancini et al., 2002; Pannekens et al., 2019; Schupp et al., 2020; Zeman et al., 2014), the community in our study appears more temperature restricted.

Some methanogenic phylotypes exhibited broader tolerance. For example, *Methanosarcina* was present at 10–40°C and is known to grow under both mesophilic (25–40°C) and thermophilic (50–55°C) conditions, utilizing either acetate or H_2/CO_2 (Jetten et al., 1992; Wagner, 2020). Other phylotypes showed more restricted temperature distributions—for instance, an unclassified member of *Methanomicrobia* lineage was only detected between 10°C and 30°C, while *Methanobacterium* was predominant at 40°C, indicating a possible temperature-specific niche. The absence of methanogenesis above 40°C reinforces the idea that the native microbial community from this contaminated site is not adapted to higher temperatures.

Implications for the ATES-bioremediation combination and outlook

In this study, we systematically assessed the effect of temperature on microbial reductive dechlorination of TCE, relevant to combining enhanced natural attenuation with ATES. At the study site, TCE and its degradation products were detected in both the aquifer (particularly in groundwater) and the surrounding sediment matrix, down to a depth of 50 meters below ground surface.

Based on our findings, within LT- ATES systems (≤25°C), reductive dechlorination of TCE to ethene is unlikely to be inhibited, as we observed dechlorination to ethene up to 30°C. However, the VC accumulation at 30°C and *cis*-DCE at 40°C deserves more attention, specifically in the context of medium temperature ATES (MT-ATES; 25–40°C), given their health and environmental impacts (Benedict et al., 2024; Williams et al., 2022).

In HT-ATES systems, where injection temperatures exceed 60°C, microbial dechlorination is likely to be inhibited. Nevertheless, *in situ* temperature gradients are expected, ranging from elevated temperatures near the injection point to cooler conditions farther from the heat source (Kallesøe and Vangkilde-Pedersen, 2019; Lerm et al., 2013). This could create subsurface zones where dechlorination is enhanced (25–30°C) and others —particularly near the hotter core—where it may stall at *cis*-DCE. Importantly, such thermal effects would primarily occur in the aquifer, while surrounding low-permeability sediments (e.g., aquitards) may remain largely unaffected and continue to act as long-term sources or microbial reservoirs. These findings underscore the importance of accounting for spatial heterogeneity in thermal effects when designing ATES–bioremediation systems.

Beyond considering an upper temperature threshold (e.g., 40°C) or modulating injection and extraction temperatures, further *in situ* investigations are essential to evaluate the long-term feasibility of the combined approach. Future studies should also evaluate seasonal temperature fluctuations via heat cycle experiments and field validations in diverse geological settings.

Applying microbiological tools such as quantitative PCR (qPCR) and metagenomics could provide deeper insights into temperature-driven shifts in community structure and function, thereby improving our understanding of subsurface microbial interactions and bioremediations under dynamic thermal conditions. Moreover, studies should assess recolonisation rates—whether from surviving consortia in sediment or from microbial inflow following heat-affected phases (Friis, 2006; Friis et al., 2007a).

If complete reductive dechlorination cannot be re-established after high-temperature exposure, biostimulation or bioaugmentation with temperature-adapted consortia could be viable options (Delgado et al., 2014; Ni et al., 2018; Sewell and Gibson, 1991; Xiao et al., 2020).

Additionally, temperature effects on microbial partners supplying essential cofactors, electron donors (e.g., hydrogen) and carbon source (e.g., acetate) must be considered, as inhibition of these auxiliary processes may limit dechlorination rates.

In summary, our results show that lower temperatures (e.g., 10°C) delayed the onset of both dechlorination and methanogenesis (Supplementary Figures S1A–D). Temperature significantly influenced microbial community composition—promoting syntrophic organisms such as fermenters and acetogens that convert lactate into key metabolites. These, in turn, are utilized by reductive dechlorinators (e.g., *Dehalogenimonas*), and competitors such as hydrogenotrophic methanogens, which compete for shared resources. The 20°C and 30°C incubations supported more favorable conditions for lactate degraders and the dechlorinators, resulting in earlier and more complete product accumulation.

Conversely, unfavorable conditions (e.g., 40°C) could inhibit or eliminate reductive dechlorinators, leading to necromass

scavenging and accumulation of intermediates like *cis*-DCE or VC. Accumulation may result from competition for hydrogen among processes such as reductive dechlorination, ethene-to-ethane reduction, and hydrogenotrophic methanogenesis, or from toxic effects of accumulated intermediates on the native community (Garcia et al., 2018; Lee and Lee, 2016; Smatlak et al., 1996).

Thus, the observed VC accumulation at 30°C and *cis*-DCE at 40°C remains a key challenge for bioremediation. In conclusion, combining LT-ATES with enhanced natural attenuation is feasible based on our results. However, maintaining a suitable operational temperature range is crucial when implementing MT- or HT-ATES at chlorinated ethene-contaminated sites to ensure a sustainable, continuous natural attenuation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

MB: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology. SD: Formal analysis, Investigation, Methodology, Writing – review & editing. CV: Writing – review & editing, Supervision. MF: Writing – review & editing, Resources. IN: Writing – review & editing, Conceptualization, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frwa.2025.1566161/full#supplementary-material

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Chapter 7.

Synthesis discussion

7.1. Integrated overview of temperature effects on anaerobic processes

This synthesis addresses the overarching aim of the thesis: to elucidate how temperature controls key anaerobic microbial processes relevant to ATES, integrating findings from Publications I–V. It provides a unified interpretation of how temperature influences microbial activity, community composition, and contaminant transformation potential in anaerobic aquifer systems. Publications I–III investigate temperature effects on the mineralisation of model carbon substrates—acetate and toluene—under sulfate-reducing conditions. These studies elucidate fundamental patterns in carbon turnover, referring to the transformation and mineralisation of organic carbon to CO_2, and in energy flow, which describes the transfer of metabolic energy through oxidation of organic substrates coupled to sulfate reduction. In contrast, Publications IV and V examine temperature effects on the transformation of chlorinated ethenes, with Publication IV offering a literature-based synthesis of temperature-dependent TCE degradation and Publication V presenting experimental evidence from controlled heating studies. Although these two research lines target different substrates and functional groups of microorganisms—sulfate reducers and dechlorinators—they are unified by a common goal: to understand how temperature variations, such as those induced by ATES, shape anaerobic microbial activity and contaminant transformation potential in groundwater systems.

7.2. Cross-study synthesis of temperature responses

Across the experimental studies (Publications I–III and V), temperature emerged as a key determinant of microbial activity and metabolic stability. The experimental work drew on sediments and long-term enrichments from three contrasting field contexts. The Zeitz and Weißandt–Gölzau materials represent shallow, hydrocarbon-impacted aquifers in central Germany dominated by anoxic, sulfidogenic conditions; Zeitz enrichments originated from UFZ on-site percolation columns operated for years with sulfate-rich, benzene-contaminated groundwater at approximately 15 °C, whereas Weißandt–Gölzau sediments were collected directly from a contaminated aquifer (8.5–9.5 m below ground surface (BGS)) and maintained under anoxic storage prior to enrichment. In contrast, the Ferrara sediments originated from a chlorinated-ethene contaminated site in northern Italy (natural groundwater temperatures ∼16–20 °C), where field evidence indicates ongoing reductive dechlorination with frequent VC accumulation. These distinct site histories and in situ temperature regimes provide the ecological context for interpreting temperature thresholds and recovery patterns across Publications I–V. Using sediments from Zeitz, Weißandt-Gölzau, and Ferrara, these studies collectively revealed that moderate warming ($12 \rightarrow 25$ °C) consistently enhanced microbial activity, whereas higher temperatures caused sharp declines or complete inhibition. Under sulfate-reducing conditions, both acetate and toluene mineralisation increased from 12 °C to 25 °C, reflecting expected rises in metabolic rates within the mesophilic range. However, further warming (>38 °C) resulted in significant inhibition of both sulfate reduction and substrate mineralisation, indicating limited thermal tolerance of mesophilic consortia. Notably, active toluene- and acetate-degrading sulfate-reducing cultures were successfully maintained at 45 °C—a transition point between mesophilic and thermophilic regimes that has rarely been reported. In particular, the acetate-mineralising sulfidogenic enrichment exhibited partial resilience after exposure to both cooler (12-25 °C) and warmer (60 °C) conditions, resuming activity upon return to 45 °C. This finding extends the known temperature limits of such communities, indicating limited but meaningful resilience to transient heat inputs in low-temperature ATES systems.

The TCE dechlorination studies (Publications IV–V) revealed a clear temperature dependency. Complete reductive dechlorination occurred up to 20 °C, while higher temperatures led to the accumulation of intermediates—VC at 30 °C, cis-DCE at 40 °C—and complete inhibition at 50 and 60 °C. These findings are consistent with the literature summarized in Publication IV, which identifies 30 °C as an approximate threshold for effective TCE

dechlorination. Methanogenesis, a related anaerobic process, exhibited slightly higher thermal tolerance, persisting up to 40 °C before being inhibited. This confirms that dechlorinating communities are more temperature-sensitive than methanogens, likely due to their narrower ecological niches and enzymatic constraints. Similar patterns have been reported elsewhere, where complete TCE dechlorination was not observed above 40 °C, even though methanogenic activity continued (Conrad, 2023).

Two recent studies further support and refine these findings. One demonstrated that dechlorination of PCE and TCE was most rapid between 25–35 °C, while complete dechlorination to ethene was most effective at 25–30 °C (Yamazaki et al., 2022). The authors observed that microbial communities—particularly *Dehalococcoides* harboring VC reductase genes—were adversely affected at 35 °C, leading to VC accumulation. Although microbial composition remained relatively stable between 15–30 °C, significant shifts occurred at 35 °C. A second study focused specifically on cis-DCE dechlorination under biostimulation at 15 and 30 °C and found that elevated temperature consistently accelerated dechlorination kinetics across samples from different sites (Yamazaki et al., 2020). This was accompanied by enhanced growth of distinct *Dehalococcoides* populations at 30 °C, suggesting a temperature-driven selection of more active dechlorinators. Interestingly, despite these functional differences, microbial communities converged to similar compositions after repeated subculturing, indicating a limited long-term impact of moderate heating on overall community structure.

Together, these findings reinforce the view that thermal enhancement up to approximately 30 °C can improve dechlorination performance without major disruption of the microbial ecosystem. However, temperatures beyond this threshold may impair complete detoxification by selectively inhibiting key organisms involved in the final steps of the dechlorination pathway.

7.3. Thermal limits, mechanistic insights, and resilience of anaerobic microbial processes

The temperature responses observed in this thesis are best understood in the ecological context of the investigated sediments. The microbial communities originated from shallow aquifers in Zeitz, Weißandt–Gölzau, and Ferrara, which are characterised by generally low to moderate and relatively stable in situ groundwater temperatures (approximately 8–20 °C, depending on site) and limited natural thermal variability. These environments host

predominantly psychrotolerant to mesophilic groundwater microorganisms that have not evolved under sustained exposure to elevated temperatures, in contrast to deep oil-reservoir communities that experience long-term heating. Consequently, warming to 20–30 °C already represents a substantial thermal shift relative to their native habitat, while exposure to 40–60 °C constitutes an extreme disturbance rather than a gradual extension of their natural niche.

Across the five studies, consistent thermal response patterns emerged among functionally distinct microbial groups: moderate warming stimulated metabolism, whereas high temperatures increasingly constrained activity. The establishment of active sulfate-reducing cultures at 45 °C provides rare evidence of adaptation near the upper mesophilic boundary, whereas the absence of activity at 60–80 °C highlights the separation between mesophilic and thermophilic regimes in the studied sediments. Comparable thermal thresholds for sulfate reduction and TCE dechlorination were observed for the shallow aquifer communities investigated here. While community composition, substrate type, and redox environment influenced rates and pathways, these factors did not markedly shift the upper temperature limits of anaerobic metabolism within this system (Chambon et al., 2013; Yoshikawa & Zhang, 2020).

Microbial community analyses showed temperature-driven shifts in dominant phylotypes, ranging from declining diversity under inhibitory conditions to "pasteurised" communities that were compositionally similar but functionally suppressed. The persistence of sulfate reduction at 45 °C suggests the proliferation of moderately thermotolerant taxa capable of sustaining activity when sensitive populations decline. In contrast, the loss of dechlorination activity above 30 °C reflects the thermal sensitivity of key reductive dehalogenases and the vulnerability of specialist lineages such as *Dehalococcoides*.

Across the studies, microbial function often persisted despite clear taxonomic turnover, a hallmark of functional redundancy (Louca et al., 2018). Together, these observations align with broader ecological frameworks suggesting that redundancy can buffer biogeochemical processes against compositional change, but that this buffering capacity is limited in systems dominated by functional specialists. It should also be noted that enrichment culturing itself imposes an additional thermal and ecological filter. Many anaerobic enrichment cultures in the literature are routinely incubated under mesophilic conditions (20–30 °C), even when derived from cold aquifers. Such incubation favours faster-growing mesophiles and may suppress cold-adapted populations, effectively pre-adapting cultures to elevated temperatures before experimental manipulation. This selection effect likely contributes to the apparent thermotolerance observed in some laboratory systems and must

be considered when extrapolating enrichment-based temperature limits to natural aquifers.

Thermal history was a critical determinant of recovery following heat exposure. Cultures acclimated at 25 °C resumed sulfate-reducing activity after exposure to 60 °C, whereas those maintained at 12 °C showed little or no rebound. This suggests that prior exposure within the mesophilic range may confer partial thermotolerance, potentially by retaining heat-tolerant subpopulations or inducing protective physiological states. Lag phases following heat stress indicate that recovery depends on both the extent of thermal damage and the pre-existing physiological state. In some cases, resilience was likely supported by endospore-forming sulfate-reducing bacteria, such as members of the genus *Desulfotomaculum*, whereas irreversible loss of key dechlorinators prevented functional rebound (Bell et al., 2020; Demin et al., 2024; Fichtel et al., 2015).

Endospores exhibit extreme resistance to environmental stress—including heat, nutrient limitation, and oxidative damage—due to a highly protective spore coat, dehydration of the core, and the presence of small acid-soluble proteins and dipicolinic acid that stabilise DNA and proteins (Dworkin & Shah, 2010; Setlow, 2007). In contaminated aquifers or sedimentary environments, these dormant spores can survive unfavourable conditions (e.g., high temperature or electron acceptor limitation) and germinate once conditions return to mesophilic—and thus favourable—states (e.g., after cooling phases in seasonal ATES operation). Such a seed bank extends the effective thermal niche of the community, enabling the re-establishment of key functions (e.g., sulfate reduction) after heat stress. This mechanism may be especially important in shallow aquifers with fluctuating temperatures, where non-spore-forming specialists (e.g., obligate dechlorinators) may be irreversibly lost under thermal stress, whereas spore-formers persist and maintain long-term biodegradation potential.

Collectively, these findings highlight that functional stability under ATES-relevant temperature fluctuations depends on (i) initial diversity, (ii) the presence of thermotolerant or spore-forming taxa, and (iii) the community's prior thermal exposure. Understanding how redundancy and thermal memory interact therefore provides a mechanistic basis for predicting when aquifer microbiomes can maintain—or lose—critical functions under dynamic thermal regimes.

The experimentally observed temperature thresholds (25–30 °C for complete TCE dechlorination and 38–45 °C for sustained sulfate reduction) therefore define the thermal operating window in which ATES can be safely coupled with natural attenuation. These limits are consistent with groundwater protection policies that restrict thermal perturbations to prevent adverse ecological impacts (Hähnlein et al., 2013). For shallow geothermal

systems in Europe, the commonly accepted groundwater temperature range lies between 5 and 25 °C, with maximum allowable temperature changes (ΔT) typically limited to 3–11 K depending on the regulatory framework (Hähnlein et al., 2013). Such regulations reflect the precautionary principle embedded in European environmental policy, which aims to prevent harm even under scientific uncertainty. Conversely, risk-based approaches, such as those applied in parts of North America, react to impacts once they are measurable, potentially permitting larger thermal excursions. Across countries, regulatory frameworks remain heterogeneous and are often inconsistently enforced, yet recent policy developments increasingly call for harmonisation to ensure sustainable subsurface energy use. Thus, limiting groundwater heating to within the mesophilic range is essential not only for microbial functionality but also for compliance with evolving environmental governance of ATES systems.

It should be emphasised, however, that elevated temperatures are not inherently detrimental to groundwater quality in all settings. In aquifers not impacted by chlorinated ethenes, warming into the upper mesophilic range or even beyond may be acceptable or even beneficial, as higher temperatures can enhance the degradation of hydrocarbons and other organic contaminants by accelerating microbial metabolism. Moreover, in high-temperature ATES (HT-ATES) systems the volume of strongly heated groundwater typically represents only a small fraction of the total aquifer, with temperatures decreasing rapidly along thermal gradients away from injection wells. Within these fringe zones, moderate warming may stimulate microbial activity and contaminant turnover rather than suppress it, particularly for substrates such as toluene or acetate that are degraded by more thermotolerant functional guilds.

7.4. Permanent versus temporary temperature effects

Persistent warming in ATES creates stable thermal gradients around warm wells (Bridger & Allen, 2014; Heldt et al., 2024) and is predicted to expand heated zones over weeks to months (Wienkenjohann et al., 2025). Minor permanent shifts within the mesophilic range (e.g., 12 → 25 °C; 45 → 38 °C) generally did not impair toluene mineralisation or sulfate reduction, despite shifts in dominant taxa. In contrast, larger temperature increases (e.g., 12 → 45 °C; 12 → 60 °C) frequently led to persistent inhibition, consistent with the loss of heat-sensitive key taxa and reduced functional redundancy.

By comparison, recent HT-ATES field tests involving injection temperatures up to 80 °C in uncontaminated aquifers reported pronounced community restructuring, including

increased abundances of *Bacillota*, suggesting selection for thermophilic or spore-forming taxa previously dormant at ambient temperatures (Keller et al., 2026). Importantly, these systems did not depend on temperature-sensitive specialist functions such as reductive dechlorination, underscoring that tolerance to extreme heating is strongly context- and function-dependent.

In natural ATES systems, thermal exposure does not occur in isolated microcosms but within a dynamically mixed groundwater environment. Pumping, reinjection, and buoyancy-driven flow redistribute both heat and microbial populations, exposing cells to spatially and temporally variable temperature histories (Ngninjio et al., 2024). Such mixing can introduce heat-tolerant or spore-forming taxa from cooler zones into warmer regions and vice versa, creating opportunities for recolonisation and recovery that are not captured in static laboratory incubations. In addition, advective mixing redistributes dissolved electron donors, electron acceptors, nutrients, and metabolic intermediates, which can transiently sustain microbial activity or intensify competition among functional guilds. These coupled processes may partly explain the persistence of some functions under transient heating, while simultaneously increasing the vulnerability of specialist populations such as obligate dechlorinators.

In contrast to sustained heating, ATES cycles (injection–storage–recovery–rest) impose short-term heat pulses lasting from days to weeks (Zhou et al., 2024). In our microcosms, 6–14 d heat pulses produced three consistent outcomes: (1) permanent inhibition following strong pulses ($\geqslant$60 °C), (2) temporary inhibition with lagged recovery at 45–60 °C, and (3) little or no effect under mild pulses ($\leqslant$38 °C). For TCE systems, rising temperature (10 $\rightarrow$ 40 °C) restructured communities, favoured methanogens at 40 °C, and led to incomplete dechlorination (cis-DCE/VC accumulation). This illustrates how competition for electron donors and the loss of specialist taxa constrain recovery following heating.

Together, these contrasting outcomes under permanent and transient heating define the temperature ranges within which key anaerobic functions—such as sulfate reduction, methanogenesis, nitrate reduction, and reductive dechlorination—can either recover or become impaired under ATES-relevant conditions. They further emphasise that both the duration and magnitude of thermal exposure—together with subsurface heat propagation, mixing, and recovery efficiency—determine whether microbial processes remain resilient, shift toward alternative pathways, or become irreversibly suppressed in thermally impacted aquifers.

7.5. Implications for ATES-impacted aquifers

From an applied perspective, the collective results from Publications I–V demonstrate that temperature fluctuations within the low-temperature ATES (LT-ATES) range (typically $\leqslant$25–30 °C in European regulation) are unlikely to disrupt anaerobic microbial processes essential for natural attenuation and may even accelerate biodegradation, particularly of toluene. This suggests that LT-ATES could be compatible—and in some cases synergistic—with enhanced natural attenuation of hydrocarbons and potentially TCE. At the upper mesophilic range (30–45 °C), however, dechlorination became increasingly incomplete, while sulfate reduction still persisted. These diverging sensitivities imply that the warm fringe of high-temperature ATES (HT-ATES) systems, where thermal gradients extend into mesophilic aquifers, represents a critical operational zone: short-term or spatially limited warming may remain tolerable for sulfate reduction, but even moderate exceedance of $\sim$30 °C can compromise complete TCE detoxification. Therefore, maintaining temperatures within the mesophilic range—preferably below the thresholds for reductive dechlorination inhibition—is essential to safeguard subsurface biodegradation processes during ATES operation.

In practice, these findings imply that the thermal management of ATES installations should not be guided by temperature criteria alone. The type and composition of contaminants present—often complex mixtures of hydrocarbons, chlorinated solvents, and their metabolites—can substantially influence microbial responses to heating. Co-occurring compounds may compete for electron donors or acceptors, inhibit key enzymatic pathways, or select for distinct functional guilds, thereby altering the overall resilience and recovery potential of the subsurface microbiome. For instance, soil microcosm experiments demonstrated that combined exposure to trichloroethylene (TCE) and toluene caused more pronounced and lasting alterations in community structure and substrate utilization patterns than either compound alone, even after contaminant removal (Fuller et al., 1997). Such interactions highlight that mixed contamination can impose persistent functional shifts, further complicating predictions of microbial activity under thermally dynamic conditions.

Consequently, the design of sustainable ATES operations should integrate both hydrogeochemical and contaminant-specific assessments to ensure that heat storage strategies do not compromise intrinsic bioremediation capacity. These insights emphasise the need for site-specific operational temperature windows that balance thermal energy efficiency with the preservation of microbial ecosystem functions—an essential consideration for sustainable ATES design and groundwater management.

7.5.1. Broader redox processes and beneficial temperature effects in ATES systems

While this thesis focuses on sulfate reduction and reductive dechlorination as key anaerobic processes governing contaminant fate, other redox pathways are also relevant for ATES-impacted aquifers. Aerobic respiration, denitrification, and Fe(III) reduction commonly occur in groundwater systems, particularly in recharge zones, mixing zones, or aquifers with fluctuating redox conditions. These processes often exhibit broader thermal tolerance than reductive dechlorination and can remain active, or even be stimulated, at elevated temperatures.

Recent work by Wang and colleagues provides evidence that some anaerobic redox processes retain functionality across a wide temperature range under nitrate-reducing conditions. In controlled aquifer microcosms, dissimilatory nitrate reduction persisted from 12 to 60 °C, with complete mineralisation of the electron donor acetate confirmed by $^{13}CO_2$ production (Wang et al., 2025). Community composition shifted systematically with temperature, with Proteobacteria dominating between 12 and 38 °C and Firmicutes—particularly Symbiobacteriaceae and Paenibacillaceae—prevailing at 45 and 60 °C. This pattern indicates functional continuity through replacement of mesophilic by thermophilic nitrate-reducing guilds rather than metabolic collapse.

Importantly, this behaviour contrasts with the narrower temperature tolerance observed here for sulfate reduction and reductive dechlorination in shallow aquifer communities, underscoring that upper thermal limits are strongly process- and community-specific. While sulfate reduction and chlorinated-ethene detoxification were constrained to the mesophilic window in the investigated sediments, nitrate reduction may remain active under substantially higher temperatures. This suggests that moderate to high warming in ATES systems can selectively suppress some microbial functions while sustaining or enhancing others.

This process-specific temperature sensitivity is also evident from our own experimental work (Bin Hudari et al., 2020). In a laboratory microcosm study using a hydrocarbon-contaminated aquifer community incubated between 12 and 80 °C, acetate mineralisation exhibited a clear redox-dependent temperature response. At 12–38 °C, acetate was mineralised both aerobically and under sulfate-reducing conditions, with sulfate-coupled mineralisation rates at 25 and 38 °C exceeding those at 12 °C by approximately a factor of six. In contrast, at 45–60 °C acetate mineralisation occurred predominantly under oxic conditions, while sulfate-reducing activity was strongly constrained, and no mineralisation

was observed at 80 °C.

These results demonstrate that aerobic heterotrophic processes tolerate higher temperatures than sulfate reduction within the same aquifer-derived microbial community. Community analyses further showed a temperature-driven replacement of mesophilic sulfate reducers by spore-forming Bacilli under oxic conditions at elevated temperatures, whereas thermophilic sulfate reducers were not detected. Together, these findings highlight that elevated temperatures in ATES systems can selectively favour aerobic or nitrate-reducing pathways while suppressing sulfate reduction, reinforcing that temperature effects on biodegradation are strongly modulated by electron acceptor availability rather than temperature alone.

In ATES systems, thermal perturbations are spatially heterogeneous. High temperatures are typically restricted to the immediate vicinity of warm wells, whereas most of the aquifer is exposed to moderate warming within thermal mixing zones. In these zones, elevated temperature can accelerate microbial metabolism, hydrocarbon degradation, nitrate reduction, and Fe(III) reduction, potentially enhancing natural attenuation rather than suppressing it. Thus, while excessive heating may locally inhibit temperature-sensitive processes, moderate warming along thermal gradients may create favourable conditions for biodegradation.

Taken together, these considerations indicate that elevated temperature in ATES systems can exert both inhibitory and beneficial effects, depending on the redox regime, contaminant type, and spatial position within the thermal plume. Sustainable ATES design should therefore aim to exploit the positive effects of moderate warming in mixing zones while avoiding prolonged exposure of temperature-sensitive processes—such as complete reductive dechlorination—to damaging thermal extremes.

7.6. Implications for ATES development and environmental risk

Our results demonstrate that microbial communities adapted to 12–25 °C can tolerate moderate temperature fluctuations, reflecting the inherent resilience of aquifer microbiota. However, resilience depends on multiple parameters—including the duration and intensity of heating, the diversity and functional redundancy of the community, and the cooling phase following disturbance. Experimental studies addressing cyclic or long-term heat storage remain scarce; the recent field work of Keller et al. (2026) highlights that repeated heating

can progressively alter community structure and reactivity. Similarly, Lerm et al. (2013) showed that temperature gradients in a deep geothermal system induced microbial shifts associated with corrosion and mineral precipitation. These findings demonstrate that even thermally tolerant microbial assemblages may undergo functional changes with system-level consequences.

In relation to policy-defined temperature limits, our findings show that activity between 12–25 °C is sustainable and interchangeable for toluene mineralisation under sulfate-reducing conditions. This aligns with European ATES regulations that typically restrict groundwater temperatures to $\leqslant$25 °C or permit only small thermal increases (ΔT of 3–11 K) above ambient conditions to safeguard ecosystem functioning (Hähnlein et al., 2013). Importantly, we established active toluene- and acetate-mineralising cultures from sediments naturally adapted to 10 °C and maintained function at 45 °C—the upper mesophilic boundary for sulfate reduction observed in our studies. Acetate-mineralising cultures also tolerated shifts between 12 °C and 60 °C, with *Desulfotomaculum* consistently present and likely responsible for sustaining activity under heat stress.

Although 45 °C exceeds regulatory limits for low-temperature ATES, such temperatures may occur transiently or locally within high-temperature ATES systems depending on injection conditions and flow path geometry (Kallesøe & Vangkilde-Pedersen, 2019; Lerm et al., 2013). Thermal modelling and field observations suggest that warm fringe zones surrounding HT-ATES wells can temporarily reach 40–45 °C before stabilising at lower temperatures during long-term operation (Gao et al., 2019; Schout et al., 2014). These observations emphasise that while sulfate-reducing processes may persist in such thermal gradients, more temperature-sensitive pathways—especially reductive dechlorination—require careful operational control to prevent impaired natural attenuation capacity.

Beyond microbial considerations, increasing subsurface temperature can mobilise organic compounds, heavy metals, and trace elements, altering groundwater geochemistry and biodiversity (Bonte et al., 2011a, 2013a; Bonte et al., 2013c; Fleuchaus et al., 2020; Jesußek et al., 2013a; Possemiers et al., 2014; Riedel, 2019; Saito et al., 2016). Elevated temperatures also lower dissolved oxygen concentrations, favoring sulfate reduction and anoxia (Riedel, 2019). Only a few *in-situ* studies have examined contaminant biotransformation under such temperature regimes—e.g., cis-DCE at 12–25 °C and TCE at 18–22 °C—highlighting how little is known about HT-ATES effects on redox-driven processes such as dechlorination (Sommer et al., 2013; Truex et al., 2007; Wienkenjohann et al., 2024).

At the scale of an ATES installation, however, thermal impacts are spatially heteroge-

neous. High-temperature zones are typically restricted to a relatively small volume around warm injection wells, whereas most of the aquifer experiences moderate temperature increases along thermal gradients. In these fringe zones, warming from ambient conditions into the upper mesophilic range can enhance microbial activity and biotransformation rates, including reductive dechlorination, even if processes are inhibited in the hottest core. Thus, system-level effects may include both local inhibition and peripheral stimulation of contaminant degradation, depending on the spatial distribution of temperatures and flow paths.

Our comparative data across sediments show that native community composition strongly determines thermal responses. Different aquifers host distinct microbial consortia, with varying capacities for adaptation or tolerance. Rare thermotolerant or thermophilic taxa may persist as "seed banks," enabling limited resilience—as seen in the acetate-mineralising 45 °C cultures that tolerated both 60 °C and 12 °C (Lennon & Jones, 2011; Wisnoski & Lennon, 2021). Diversity therefore underpins sustainable ecosystem function and recovery potential under thermal stress (Botton et al., 2006; Finlay et al., 1997; Garcia et al., 2018). Loss of diversity can erode functional redundancy, compromising ecosystem integrity and slowing recovery after thermal perturbation (Delgado-Baquerizo et al., 2016). Consequently, ATES operation should adopt site-specific temperature limits based on native aquifer characteristics rather than universal thresholds.

7.7. Broader perspective

By integrating experimental and literature-based data, this synthesis provides a unified understanding of how temperature governs anaerobic microbial processes relevant to ATES. The results demonstrate that while mesophilic aquifer communities involved in carbon turnover—such as sulfate reducers—can adapt to moderate warming, those mediating chlorinated-ethene transformation exhibit narrower temperature ranges and limited recovery once thermal thresholds are exceeded. These upper boundaries constrain long-term functionality, particularly for specialist dechlorinators under ATES-relevant conditions.

Thermophilic reductive dechlorination has been demonstrated only in a few specialised systems, such as thermophilic enrichments from PCE-contaminated harbour sediment (Kengen et al., 1999) and enzymatic dechlorination of 1,2-dichloroethane at 60 °C (Holliger et al., 1992). These exceptional cases suggest that thermophilic dechlorinating metabolisms could emerge under strong selective pressures in hot, contaminated environments, implying possible site-specific evolution of temperature tolerance at HT-ATES installations. How-

ever, such observations remain rare, and our results—along with most field data—indicate that effective chlorinated-ethene remediation currently lies within the mesophilic window.

Despite pathway-specific differences, the overall consistency in thermal thresholds across functional guilds highlights temperature as a cross-cutting control on both carbon and contaminant biotransformation in groundwater, and therefore a key aspect that must be taken into account in the design and operation of ATES systems.

Future research should therefore prioritise assessing the long-term recovery and adaptation potential of aquifer microbiota following transient heat exposure, alongside identifying microbial lineages or consortia that confer thermotolerance within otherwise mesophilic communities. Such work will be essential for developing predictive, microbially informed models of ATES operation that safeguard biogeochemical functions under changing thermal regimes.

Collectively, these insights underline the importance of integrating microbial ecology into the design and operation of ATES systems. While this synthesis defines the current understanding of temperature-dependent processes and thresholds, it also exposes critical gaps regarding long-term resilience, recovery dynamics, and field-scale validation. The following chapter therefore outlines key methodological limitations and future research directions needed to translate these findings into practical, environmentally responsible ATES applications.

Summary and outlook

Together, Publications I–V demonstrate that mesophilic shallow-aquifer communities tolerate moderate thermal variation but face upper functional limits near 45 °C for sulfate-reducing mineralisation and 30 °C for complete TCE dechlorination. Community resilience arises from functional redundancy and thermal memory, yet permanent heating can drive irreversible compositional shifts and loss of specialist functions. Thermophilic anaerobic hydrocarbon degradation at much higher temperatures has been documented in deep oil-reservoir systems, but these involve long-adapted microbial communities that differ fundamentally from shallow aquifers. For ATES design, operating within site-specific, microbially informed thresholds can expand storage capacity while safeguarding natural attenuation. The implications of these findings for future research directions and ATES system design are developed further in the following chapter.

Chapter 8.

Future perspectives and outlook

8.1. Methodological limitations

Most results presented in this thesis are derived from microcosm incubations—closed systems that cannot fully replicate open aquifer conditions, including recharge, advection, dispersion, and mixing. As a result, direct extrapolation to field settings is inherently limited. Nonetheless, several laboratory-derived biodegradation patterns have been verified under pilot- and field-scale conditions through combined in situ and laboratory approaches (Bahr et al., 2015). Further research, particularly temperature-cycling experiments, is needed to evaluate the aquifer microbiome's capacity to recover after exceeding putative thermal thresholds. Ecosystem resilience is a critical consideration in HT-ATES risk assessment: if injection temperatures are too high, native microbial community structures may fail to re-establish even after ambient conditions are restored. Although not a definitive threshold, sustained temperatures near 50 °C are considered a critical stressor in microbial ecology, with the potential to disrupt diversity and impair community recovery under disturbance (Lennon & Jones, 2011; Philippot et al., 2021; Wisnoski & Lennon, 2021).

Beyond changes in diversity, temperature management can also alter redox zonation, with potential repercussions for ecosystem function (Bonte et al., 2013a; Griebler et al., 2016; Jesußek et al., 2013a). Studies have shown that temperature influences the positioning and extent of redox boundaries in subsurface environments, which in turn affects the biogeochemical processes governing contaminant attenuation. For example, warmer conditions can shift redox zonation along infiltration paths, altering the effectiveness of natural attenuation mechanisms. Additionally, combined redox and temperature shifts have been demonstrated to modulate the degradation of pharmaceuticals and other pollutants, while broader ecological theory highlights how environmental fluctuations—such as temperature pulses—can reshape microbial redox behavior and functional potential (Burke et al., 2014;

Nguyen et al., 2021). Sulfate reduction tends to dominate at higher temperatures (>25,°C), while iron reduction prevails at lower temperatures (Bonte et al., 2013a). Methanogenesis, in turn, typically occurs within the mesophilic range (approximately 20–30,°C) and may partly overlap with sulfate reduction under ATES-relevant conditions, particularly in zones where electron donors are abundant and sulfate becomes limiting (Bonte et al., 2013a; Jesußek et al., 2013b).

Further methodological constraints include: (i) the duration of thermal exposure (short pulses versus long-term plateaus); (ii) limited representation of aquifer heterogeneity (where low-permeability layers may act as microbial reservoirs); and (iii) molecular resolution—most experiments relied on amplicon sequencing, whereas metatranscriptomics or proteomics would more precisely identify active pathways. Moreover, current microcosms cannot capture recolonisation via advective inflow after heat perturbations.

8.2. Key empirical insights informing future work

The experiments presented in this thesis collectively demonstrate that temperature governs both the rate and completeness of anaerobic degradation processes, with distinct thresholds for sulfate reduction and reductive dechlorination. These results underscore the need to consider temperature not merely as a physical boundary condition but as a biological driver shaping the structure and function of aquifer microbiomes.

Recent work on temperature-resilient denitrification in aquifer systems illustrates that some anaerobic redox pathways can maintain functionality under thermal regimes that inhibit sulfate reduction or reductive dechlorination (Qu et al., 2022; Wang et al., 2025). Such findings highlight the need to move beyond single-process assessments and to explicitly compare pathway-specific thermal sensitivities when evaluating the biogeochemical impacts of ATES-induced heating.

Future research should focus on quantifying how transient temperature pulses—such as those induced by ATES operation—affect the fluxes of redox equivalents and the resulting accumulation of intermediates that influence syntrophic interactions among fermenters, dechlorinators, and methanogens. Integrating stable isotope tracing, transcriptomic profiling, and reactive transport modelling could help distinguish reversible heat-induced inhibition from irreversible functional loss.

A further empirical priority is to evaluate recovery dynamics under repeated heating–cooling cycles, reflecting realistic ATES operation. Particular attention should be given to competition among microbial guilds for shared substrates (e.g., hydrogen, acetate)

and to the role of microbial seedbanks in re-establishing activity following disturbance. Linking these laboratory insights with in situ monitoring data will enable a mechanistic understanding of how thermal stress and contaminant mixtures jointly control biodegradation efficiency in thermally influenced aquifers.

Future research should also explicitly evaluate the potential benefits of moderate warming in ATES systems. In thermal mixing zones surrounding injection wells, elevated temperatures may enhance reaction kinetics, stimulate hydrocarbon degradation, and increase the rates of denitrification and Fe(III) reduction, thereby improving natural attenuation of mixed contaminant plumes. Identifying the temperature windows in which such positive effects outweigh the risks to temperature-sensitive processes, such as complete reductive dechlorination, will be critical for optimising ATES operation in contaminated aquifers.

8.3. Research needs and future directions

8.3.1. From microcosms to field cycles

Future work should bridge the gap between controlled laboratory studies and the complex dynamics of field-scale ATES operation. While microcosm experiments provide valuable mechanistic insights, they cannot fully represent hydrological processes such as recharge, advection, and dispersion that influence temperature propagation and microbial responses in situ. Laboratory column studies using native sediment and groundwater offer a practical intermediate step, as they enable flow-through conditions and thermal gradients to be investigated under controlled yet hydraulically realistic settings. Where field experiments or pilot installations are not feasible, such systems can therefore help validate laboratory observations and refine model assumptions.

Nevertheless, field-scale studies remain essential to confirm laboratory findings under realistic geological and operational conditions. These should integrate long-term thermal monitoring with microbial and geochemical characterisation to determine how repeated heating and cooling cycles influence both community resilience and contaminant turnover. Numerous hydrogeological models simulate thermal plumes and recovery dynamics, but few explicitly incorporate microbial processes or verify predictions through in situ observations. Linking these physical and biological dimensions will be crucial for developing predictive frameworks of microbial stability and function under cyclic ATES conditions.

8.3.2. Thermal cycling fatigue and spatial heterogeneity

Long-term thermal cycling experiments are needed to determine whether repeated heating and cooling can sustain microbial activity, or whether "thermal fatigue" causes progressive functional decline. Spatial heterogeneity will strongly moderate these dynamics. Thermal impacts are most pronounced in highly permeable aquifer zones, where advective heat transport and microbial turnover are greatest. In contrast, low-permeability layers (aquitards) experience slower heat diffusion, remaining comparatively cooler and acting as thermal buffers. Such zones may serve as microbial refugia that preserve community diversity and facilitate recolonisation of previously heated regions once temperatures return to mesophilic levels.

To better resolve these spatial effects, laboratory column systems that incorporate intact sediments and natural groundwater offer a promising intermediate step between batch microcosms and field-scale pilot tests. Controlled thermal gradients in such setups would allow mechanistic evaluation of microbial migration, resilience, and recovery in physically realistic aquifer architectures. The spatial variability of temperature, permeability, and redox conditions will thus govern both the extent of thermal disturbance and the potential for ecological renewal within ATES-impacted aquifers.

8.3.3. Functional window for degraders

Whether microbial communities can sustain activity under elevated temperatures is of particular relevance for thermally influenced shallow aquifers. The ability of hydrocarbon-degrading groundwater communities to remain active within the 45–60 °C range under sulfate-reducing conditions remains uncertain, since thermotolerance at such temperatures has mainly been demonstrated for other anaerobic metabolisms (e.g., iron or nitrate reduction; (Kieft et al., 1999; Slobodkin, 2005; Wang et al., 2025; Zavarzina et al., 2024)). In contrast, chlorinated-ethene-transforming consortia generally lose activity well below this range, reflecting the narrower physiological tolerance of specialised organohalide-respiring bacteria. Thermophilic anaerobic hydrocarbon degradation at much higher temperatures (up to at least 80 °C) has been documented in deep oil-reservoir systems, but these involve long-adapted microbial communities that differ fundamentally from those in shallow aquifers. Clarifying these process-specific and system-dependent temperature boundaries will be essential for predicting contaminant attenuation potential in aquifers subjected to prolonged or repeated heating.

8.3.4. Molecular tools and functional diagnostics

Applying advanced molecular techniques such as quantitative polymerase chain reaction (PCR) (qPCR), metagenomics, and metatranscriptomics would substantially improve understanding of temperature-driven shifts in microbial structure and function. These approaches can elucidate the roles of syntrophic or supporting organisms that provide essential cofactors or electron donors, such as hydrogen or acetate, thereby sustaining key anaerobic processes. Future studies should also investigate recolonisation dynamics—both from surviving consortia and through microbial inflow once temperature conditions return to ambient levels (Colwell et al., 1997; Guerrero et al., 2005; Sutton et al., 2015).

8.3.5. Interventions when function is lost

When microbial activity is irreversibly inhibited by high-temperature exposure, targeted interventions may be required to restore function. Biostimulation—through the addition of suitable electron donors, nutrients, or buffering agents—can promote recovery of surviving populations, whereas bioaugmentation with temperature-adapted consortia may reintroduce key metabolic functions under elevated or fluctuating thermal regimes (Curiel-Alegre et al., 2022; Delgado et al., 2014; Sewell & Gibson, 1991; Tyagi et al., 2011; Xiao et al., 2020). In both cases, temperature effects on syntrophic partners should be considered, as inhibition of these supporting organisms can limit overall process efficiency and delay ecosystem recovery.

8.4. Operational guidance for ATES design and monitoring

8.4.1. Temperature set-points and ramping

Results from this work support a conservative operational envelope for preserving anaerobic functionality in *contaminated aquifers*: maintaining temperatures below 25–30 °C ensures complete TCE dechlorination, while sulfate-reducing mineralisation can persist up to approximately 45 °C. Such conditions are most relevant for low- to medium-temperature ATES (LT-/MT-ATES) systems, where balancing energy efficiency with microbial integrity and groundwater protection is critical.

For HT-ATES, where operational targets often exceed 60 °C, significant shifts in microbial community composition and activity are likely in shallow aquifers, and inhibition

of mesophilic processes such as complete TCE dechlorination is therefore expected. In *uncontaminated aquifers*, however, where temperature-sensitive specialist functions are not required to maintain ecosystem services, higher operational temperatures may be acceptable. Field studies have demonstrated pronounced community restructuring under HT-ATES conditions without loss of overall microbial activity, reflecting functional replacement by thermophilic or spore-forming taxa rather than ecosystem collapse.

Thermophilic anaerobic metabolisms—including sulfate reduction and hydrocarbon degradation—are known to persist at much higher temperatures in deep oil-reservoir systems and selected field experiments, but these involve microbial communities long adapted to sustained heating. In shallow aquifers not pre-adapted to elevated temperatures, HT-ATES design should therefore prioritise spatial thermal zoning, controlled temperature ramping, and recovery phases that allow recolonisation from cooler regions, thereby maintaining system-wide resilience while minimising ecological risk.

8.4.2. Monitoring plan

Coupled geochemical and microbiological monitoring is recommended to evaluate both system performance and ecosystem health in contaminated aquifers. Key parameters should include electron donors and acceptors, intermediate products, and representative microbial groups involved in contaminant biodegradation, such as *Dehalococcoides*, *Dehalogenimonas*, sulfate reducers including *Desulfotomaculum*, and methanogens. Establishing a "basket" of indicator species and diagnostic measurements—such as changes in CO_2 or sulfide production rates, accumulation of intermediates (e.g., DCE, VC), or decline of functional genes (e.g., *rdhA*, *dsrAB*)—can provide early warning of stress or loss of function.

Monitoring lag phases following heat pulses can further indicate "thermal memory" and resilience potential, helping to identify when intervention or rest phases are needed to allow microbial recovery. Regular sampling across operational stages—during injection, storage, and recovery—should be implemented to capture transient responses and ensure early detection of functional decline. For pristine aquifers, monitoring targets may differ and should instead focus on indicators of natural biogeochemical functioning and the protection of native ecosystem services (Saccò et al., 2024).

8.4.3. Risk-graded deployment

Clean and contaminated aquifers should be managed differently. Higher temperatures may be acceptable in uncontaminated sites, whereas chlorinated-ethene–impacted aquifers require cooler regimes and contingency strategies (e.g., bioaugmentation) if degradation intermediates accumulate. In aquifers impacted primarily by hydrocarbons or nitrate, however, moderate warming can be beneficial, as elevated temperatures accelerate microbial metabolism, enhance reaction kinetics, and can stimulate processes such as sulfate reduction, denitrification, and hydrocarbon degradation, which are often more robust than complete chlorinated-ethene detoxification in urban aquifers. Risk-graded ATES deployment should therefore distinguish between sites where heating primarily poses a risk to temperature-sensitive processes and those where warming may enhance natural attenuation.

8.5. Environmental and health risk considerations

Elevated subsurface temperatures may alter groundwater quality and ecological functioning, raising concerns for both environmental protection and human health (Bonte et al., 2011b, 2011a, 2013a, 2013b; Bonte et al., 2013c; Brons et al., 1991; Jesußek et al., 2013a; Lüders et al., 2020). However, only a few *in situ* studies have investigated how thermally influenced aquifers respond in terms of contaminant fate and by-product formation—for example, the enhanced biodegradation of cis-DCE and TCE under heating (Nemecek et al., 2018; Ni et al., 2015; Truex et al., 2007). The scarcity of empirical data under realistic HT-ATES conditions limits the ability to assess risks related to contaminant mobilisation, transformation pathways, or the emergence of potentially toxic intermediates.

Combined environmental stressors—such as elevated temperature and oxygen depletion—may induce a viable-but-non-culturable (VBNC) state in bacteria including *Bacillus* and *Pseudomonas*, some of which include opportunistic human pathogens (Li et al., 2014; Pazos-Rojas et al., 2023; Zhao et al., 2017). Temperatures within the 30–40 °C range can favour the proliferation of several pathogenic species, while *Legionella pneumophila* is known to survive exposure to temperatures up to approximately 67 °C and may enter a VBNC state under heat stress (Cervero-Aragó et al., 2019; Mendis et al., 2015). As VBNC cells remain metabolically active yet undetectable by cultivation-based techniques, such heat-induced shifts in microbial viability represent both a monitoring challenge and a groundwater quality concern, particularly in potable aquifers. Although these risks are less critical for systems solely operated for energy storage, they highlight broader

ecological and public health implications of subsurface thermal perturbation. Continuous monitoring of redox dynamics, metal mobilisation, and microbial viability is therefore essential to mitigate ecological risks and ensure that ATES operation remains compatible with groundwater protection objectives.

Ultimately, the environmental integrity of ATES systems must be balanced with societal acceptance, economic feasibility, and regulatory compliance—issues that are becoming increasingly important as subsurface energy storage expands across Europe.

8.6. Societal, economic, and regulatory considerations

ATES deployment has expanded rapidly since its introduction in the 1960s, with the highest concentration of installations and research occurring in the Netherlands and Central Europe. In recent years—particularly 2024—the number of ATES-related publications has reached record levels (Marojević et al., 2025).

Raising the temperature threshold for LT-ATES could significantly increase storage capacity and system efficiency. However, societal acceptance depends on transparent communication of the associated trade-offs, such as temperature-driven shifts in microbial activity and diversity in strongly heated core zones—as suggested by recent field and pilot-scale studies—versus the preservation of ecosystem services in cooler peripheral areas. The transition from LT-ATES to HT-ATES must therefore remain both economically viable and environmentally responsible, guided by robust and transparent regulatory frameworks.

At the scale of an ATES installation, strongly heated zones typically occupy only a limited fraction of the total aquifer volume, whereas most groundwater is exposed to moderate temperature increases within thermal mixing zones. Field investigations and coupled experimental–numerical studies of ATES and HT-ATES systems demonstrate that subsurface heat transport is governed not only by advective flow along injection–extraction pathways, but also by transverse dispersion, aquifer heterogeneity, and thermally induced density-driven convection. Together, these processes promote hydraulic and thermal mixing between injected water and ambient groundwater, leading to spatially heterogeneous temperature distributions rather than uniform heating.

These transition zones can experience enhanced microbial activity and contaminant biotransformation due to elevated reaction kinetics, particularly within the upper mesophilic range. This system-scale perspective is consistent with environmental assessment frameworks proposed by the German Federal Environment Agency (UBA), which emphasise that the impacts of subsurface heat storage should be evaluated across the entire thermal

plume—including mixing zones—rather than inferred solely from peak temperatures in strongly heated core regions (Dahmke et al., 2025; Heldt et al., 2024). Assessing the balance between localised high-temperature impacts and potential system-wide benefits in moderately warmed mixing zones is therefore critical for evaluating the overall sustainability of ATES deployment.

Establishing site-specific temperature limits based on microbiological and hydrogeochemical evidence is preferable to universal thresholds, as it allows for tailored management that reflects local aquifer characteristics, contaminant loads, and ecological sensitivities. Integrating such knowledge into planning and permitting processes will be crucial for the sustainable and publicly accepted expansion of ATES systems across Europe.

Looking ahead, the long-term success of ATES will depend on integrating these societal, economic, and regulatory dimensions with robust scientific monitoring and adaptive management. Pilot-scale demonstrations and continuous field validation are needed to translate laboratory insights into operational practice, ensuring that future ATES deployment achieves both energy efficiency and environmental protection.

8.7. Future outlook

Seasonal ATES operation inherently introduces thermal fluctuations that influence both geochemical and microbial processes within aquifers. The long-term implications of repeated heating cycles, particularly within the 40–45 °C range, warrant detailed pilot-scale investigations that couple microbiological and geochemical monitoring. Combining LT-ATES with enhanced natural attenuation appears feasible based on the findings of this work; however, the implementation of medium- or high-temperature ATES (MT-/HT-ATES) at chlorinated-ethene–impacted sites will require lower operational temperatures and active monitoring to prevent the accumulation of intermediates such as DCE and VC. Where specialist dechlorinators such as *Dehalococcoides* are lost, targeted bioaugmentation or biostimulation strategies may be necessary to restore function.

Summary and final conclusions

This thesis demonstrates that aquifer microbial communities exhibit both resilience and vulnerability to temperature fluctuations. Moderate warming can accelerate anaerobic bioprocesses, but for the mesophilic shallow-aquifer communities studied here, upper functional limits emerged—around 45 °C for sulfate-reducing mineralisation of toluene

and 25–30 °C for complete TCE dechlorination. These thresholds reflect the ecological constraints of shallow groundwater systems exposed to limited natural thermal variability.

Functional redundancy and thermal memory supported recovery after short-term heating, whereas prolonged exposure caused irreversible compositional shifts and loss of specialist functions. Importantly, elevated temperature is not inherently detrimental at the system scale. In thermal mixing zones, moderate warming can enhance reaction kinetics and stimulate processes such as hydrocarbon degradation, denitrification, and Fe(III) reduction, potentially strengthening natural attenuation where chlorinated ethenes are absent or secondary. The principal risk identified here is the temperature sensitivity of specialist dechlorinators, which can lead to incomplete detoxification (cis-DCE/VC accumulation) when temperatures exceed the upper mesophilic range.

By bridging microbiology, environmental engineering, and subsurface energy storage, this work contributes: (i) experimentally constrained temperature limits for key anaerobic processes; (ii) a mechanistic understanding of microbial resilience and pathway shifts under heat stress; and (iii) a microbially informed operational framework for sustainable ATES design.

Moving forward, integrating long-term field monitoring with molecular analyses will be crucial for validating laboratory findings and establishing site-specific temperature criteria. Embedding microbial thresholds into ATES management can enable efficient subsurface energy storage while safeguarding groundwater quality and the ecological functions of aquifer microbiomes.

Appendix A.

Analytical and methodological details

This appendix summarises the analytical workflows and complementary methodological considerations applied throughout this thesis. It includes detailed descriptions of stable isotope, chromatographic, and molecular analyses, along with notes on conceptual approaches such as stable isotope probing and redox management that informed experimental design and data interpretation.

A.1. Stable isotope tracing by GC–IRMS

I quantified mineralisation of [^{13}C]-labelled substrates (acetate, α-toluene) as $^{13}CO_2$ using continuous-flow GC–IRMS on the VPDB scale (Sessions, 2006; Sherwood et al., 2013; Werner & Brand, 2001). Headspace gases were separated on a capillary GC with helium carrier, passed through an oxidation reactor to convert carbon species to CO_2, dehumidified, and introduced into the IRMS ion source. Molecular ion beams at $m/z = 44, 45$, and 46 were recorded on Faraday cups; isotopic ratios were normalised to a CO_2 reference and reported as δ^{13}C (‰, VPDB). Sample introduction was automated (or performed manually using gastight syringes) following standard protocols (Leckrone & Hayes, 1998; Merritt et al., 1995).

Fractional conversion to CO_2 (integrated mineralisation; IN, %) followed Dorer et al., 2016:

$$\mathrm{IN}(\%) = \frac{[\mathrm{HCO_3^-}]\, R_{\mathrm{VPDB}}\, (\delta_{\mathrm{end}} - \delta_{\mathrm{start}})}{n\,[\mathrm{substrate}]\, \left(1 + R_{\mathrm{VPDB}}(1 + \delta_{\mathrm{start}})\right)} \times 100,$$

where $[\mathrm{HCO_3^-}]$ is bicarbonate concentration, [substrate] the added acetate or toluene concentration, R_{VPDB} the VPDB ^{13}C/^{12}C ratio, δ_{start} and δ_{end} the initial and final headspace δ^{13}C, and n the number of labelled carbons (2 for fully labelled acetate; 1 for [^{13}C]-α-toluene).

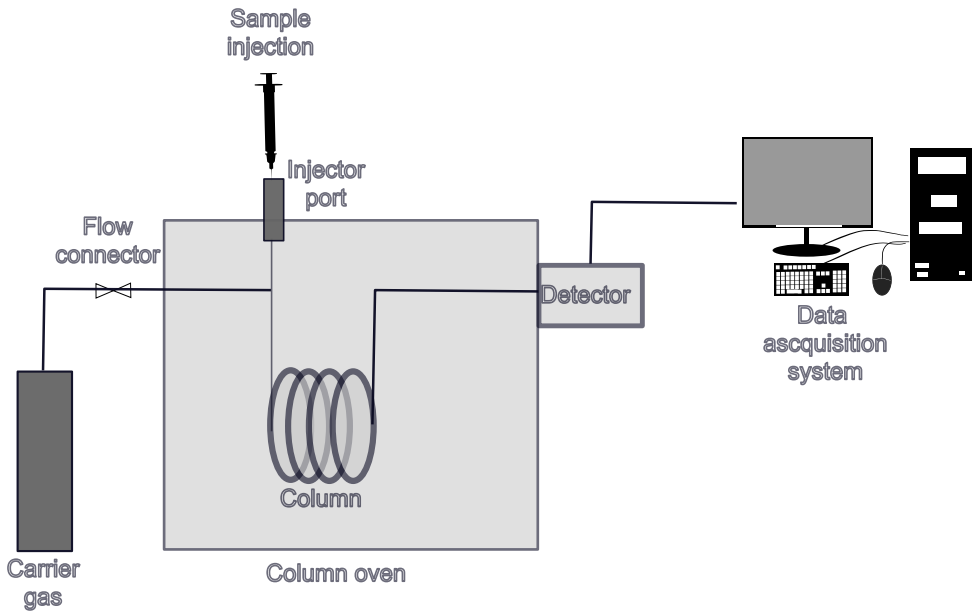


Figure A.1.: Simplified schematic of gas chromatography with flame ionization detection (GC-FID), showing sample injection, column separation in a temperature-controlled oven, and detection by flame ionization. Adapted from Soria et al. (2014) (Soria et al., 2014).

A.2. Sulfide quantification (methylene blue method)

Dissolved sulfide was quantified spectrophotometrically using the methylene blue method (Cline reagent) (Cline, 1969; Müller et al., 2009). Briefly, 50–200 μL of sample were preserved in 1 mL of 3% zinc acetate, diluted with deionised water, reacted with N,N-dimethyl-1,4,-diphenylenediammonium dichloride (DMPD) and $FeCl_3$ in 25% HCl (Cline reagent), incubated (20 min, dark), and read at 670 nm in 1 cm cuvettes. Calibration curves covered the sample range; samples exceeding linearity ($\gtrsim$1 mM) were diluted (recording dilution factors). This method offers high sensitivity and straightforward standardisation across low μM to mM sulfide.

A.3. Chlorinated ethenes and gases by GC-FID

TCE, DCE, VC, ethene, ethane, and methane were quantified by GC–FID from headspace samples. Standards were prepared in identical serum bottles and liquid volumes as experimental microcosms to ensure consistent gas–liquid partitioning. Headspace aliquots were withdrawn with gastight syringes into He-flushed vials and injected with splitless or low-split settings. Peaks were identified by retention time and quantified by peak

area against compound-specific calibration curves (multiple levels, R^2>0.99). Method performance, including limits of detection, limits of quantification, and repeatability, was routinely verified (McNair & Miller, 1998; Otson & Williams, 1982; Soria et al., 2014).

A.4. Amplicon sequencing and community analysis

Deoxyribonucleic acid (DNA) was extracted from pelleted sediments or biomass using a soil DNA kit (e.g., MoBio PowerSoil) per manufacturer instructions, eluting in 30 μL. Library preparation followed the MiSeq SOP: primary PCR with primers targeting the V4 region of the 16S rRNA gene for Bacteria and *mcrA* for methanogens, addition of Illumina adapters and dual indices (index PCR), magnetic-bead cleanup, Qubit quantification, equimolar pooling (4 nM), and storage at -20 °C prior to sequencing. Amplicons were run on an Illumina MiSeq with appropriate read length and chemistry. Processing used QIIME 2: demultiplexing, quality trimming, chimera removal, ASV inference (e.g., DADA2), and taxonomic classification against SILVA; non-bacterial/non-target reads were filtered (Caporaso et al., 2010; Hall & Beiko, 2018). Downstream analyses (alpha/beta diversity, differential abundance) followed standard workflows (parameters in the associated Methods chapter).

A.5. Stable isotope probing (conceptual and operational use)

Where indicated, stable isotope probing (SIP) was applied conceptually to interpret how substrate utilisation relates to active taxa. In strict anaerobes, singly labelled [^{13}C]-α-toluene often yields higher specific incorporation than fully labelled toluene (Bombach et al., 2009). Prior SIP studies in aquifers and soils highlight frequent roles for Betaproteobacteria, Deltaproteobacteria, and Peptococcaceae, supported by an accessory microbiome facilitating syntrophy (Kleinsteuber et al., 2012; Kunapuli et al., 2007; Laban et al., 2015; Sun & Cupples, 2012; Sun et al., 2014; Van der Zaan et al., 2012; Vogt et al., 2016).

In this thesis, SIP was used operationally only to quantify process rates through GC–IRMS measurements of labelled CO_2. Microbial community composition was assessed independently via 16S/*mcrA* amplicon sequencing. DNA-based SIP was initially considered to directly link substrate utilisation to specific taxa; however, community shifts and isotopic signals were already well resolved with the approaches used here. Addition-

ally, DNA yields from several incubations were only sufficient for amplicon sequencing, whereas nucleic-acid SIP typically requires substantially higher biomass for reliable separation of labelled and unlabelled fractions. For systems with greater microbial abundance or where functional assignments remain ambiguous, DNA-SIP could provide a valuable complement in future studies.

Appendix B.

Author contributions

The listed author contributions for each chapter corresponding to a publication follow the CRediT taxonomy.

Chapter 2 – Effect of temperature on acetate mineralization kinetics and microbial community composition in a hydrocarbon-affected microbial community during a shift from oxic to sulfidogenic conditions

Mohammad Sufian Bin Hudari: Methodology, Validation, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing.

Carsten Vogt: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

Hans-Hermann Richnow: Conceptualization, Writing - Review, Funding acquisition.

Chapter 3 – Sulfidic acetate mineralization at 45°C by an aquifer microbial community: key players and effects of heat changes on activity and community structure

Mohammad Sufian Bin Hudari: Methodology, Validation, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing.

Carsten Vogt: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

Hans-Hermann Richnow: Writing - Review, Funding acquisition.

Chapter 4 – Sulfidic toluene mineralization by aquifer microbial communities at different temperatures

Mohammad Sufian Bin Hudari: Conceptualization, Methodology, Investigation, Validation, Visualization, Writing - Original Draft, Writing - Review & Editing.

Carsten Vogt: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

Chapter 5 – Effect of temperature on microbial reductive dehalogenation of chlorinated ethenes: a review

Mohammad Sufian Bin Hudari: Methodology, Data curation, Visualization, Writing - Original Draft, Writing - Review & Editing.

Hans-Hermann Richnow: Conceptualization, Writing - Review, Funding Acquisition.

Carsten Vogt: Conceptualization, Writing - Review & Editing, Funding Acquisition.

Ivonne Nijenhuis: Conceptualization, Writing - Review & Editing, Supervision.

Chapter 6 – Temperature-associated effects on methanogenesis and microbial reductive dechlorination of trichloroethene in contaminated aquifer sediments

Mohammad Sufian Bin Hudari: Methodology, Validation, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing.

Sushmita Deb: Methodology, Validation, Investigation, Writing - Review.

Carsten Vogt: Conceptualization, Writing -Review & Editing, Funding Acquisition.

Maria Filippini: Resources, Writing - Review.

Ivonne Nijenhuis: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding Acquisition.

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