Role of microbial processes in arsenic cycling

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Summary

Arsenic (As) is a toxic metalloid of geogenic origins that can lead to serious groundwater contamination in many places worldwide. Arsenic-contaminated waters can be relatively easily treated, therefore the presence of As in drinking water is not a threat for the developed countries. However, inhabitants of developing countries, particularly in South and Southeast Asia, the most populated regions of the world, are particularly affected as the access to water treatment facilities is limited and many people, mainly in rural areas still rely on shallow groundwater wells. Moreover, many aquifers along the prime river deltas of South and Southeast Asia, such as those in the Red River, Mekong and West Bengal, are characterized by reducing conditions what triggers reductive dissolutions of As from Fe(III) minerals abundantly present in sediments. As a consequence, the release of As from sediments to groundwater led to what has been described as the 'worst mass poisoning of the human population in history', that has discriminately affected the poorer populations.

Various mechanisms of As release to the groundwater have been suggested to date. Besides reductive dissolution, changes towards high pH values, a mobilization of sorbed As by sorption competition with phosphates introduced through intensive fertilizer application, and oxidation of arsenic-bearing sulfide minerals have also been proposed. Changes in the sorption capacity of Fe(III) (oxyhydr)oxides were also suggested to play a role in As mobilization. Yet, the most commonly accepted mechanism is, that As is released from aquifer sediments during microbially-mediated reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals. This process, however, requires the presence of bioavailable carbon (C), e.g. short-chain fatty acids, that Fe(III)-reducing bacteria such as *Geobacter* spp. need as energy, electron and carbon sources for their activity and growth.

Although our understanding of the biogeochemical processes involved in As cycling has improved in the past two decades, there are still many questions that remained unanswered, specifically about the role of biological processes and the involvement of various microorganisms that can affect the fate of As in groundwater. Therefore, in this PhD project focusing on As-contaminated aquifers in Van Phuc near Hanoi (Vietnam), several experiments were conducted in order to understand how different types of organic carbon affect As mobilization from sediments, what is the role of CH_4 in As cycling and how the indigene microbial community can influence As concentration in groundwater.

In the second chapter of my thesis we explore the effect of natural organic matter (NOM) on As mobilization. Natural organic matter can contribute to As mobilization as electron donor for a microbially-mediated reductive dissolution of As-bearing Fe(III) (oxyhydr)oxides. Most laboratory studies, however, used simple fatty acids or sugars, often at relatively high concentrations, instead of naturally-occurring OM. We therefore extracted *in-situ* OM from the upper aquitard (clayey silt) and lower sandy aquifer sediments in Van Phuc (Hanoi/Vietnam), characterized its composition using various methods (FTIR, NMR, EEM and Pyrolysis GC/MS), and used 100-day microcosm experiments with aquifer material to determine the effect of *in-situ* OM on Fe(III) mineral reduction, As mobilization and the microbial community composition. We found that OM extracted from the clayey silt (OMC) aquitard resembles young, not fully degraded plant-related material, while OM from deeper sandy sediments (OMS) contains more carboxyl groups stemming from amino and fatty acids. Although all microcosms were amended with the same amount of C (12 mg C/L), the extent of Fe(III) reduction after 100 days was highest with acetate/lactate (43±3.5%) followed by OMS (28±0.3%) and OMC (19±0.8%). In contrast, although initially more As was mobilized in the acetate/lactate setups, after 100 days, similar or even higher amounts of As (8.3±0.3 and 8.8±0.8 µg As/g sediment) were mobilized by OMC and OMS, respectively, compared to lactate/acetate-amended setups (6.3±0.7 µg As/g sediment). 16S rRNA amplicon sequence analyses revealed that acetate/lactate mainly enriched *Geobacter*, while *in-situ* OM supported growth and activity of a more diverse microbial community. Our results suggest that although the *in-situ* OM is less efficient in stimulating microbial Fe(III) reduction than highly bioavailable acetate/lactate, it ultimately has the potential to mobilize the same amount or even more As.

In the third chapter I present methane (CH_4) as a driver for As mobilization. In many As-contaminated aquifers, high concentrations of CH_4 were reported, yet the role of CH_4 for As mobilization remained completely unexplored. Here, we demonstrate that CH_4 functions as an electron donor for methanotrophic microorganisms and triggers the reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals, leading to As mobilization. In microcosms with As-bearing sediments from the Red River Delta amended with environmentally relevant concentrations of CH_4, we found that CH_4 triggers Fe(III) mineral reduction, supports the growth and activity of type-1 aerobic methanotrophs and archaea affiliating with *Candidatus* Methanoperedens, increases the abundance of methane oxidation *mcrA* and *pmoA* genes, and ultimately mobilizes significant amounts of As into the water. Therefore, our study provides

evidence for a completely new mechanism of As mobilization and suggests that CH_4-driven As mobilization may occur worldwide contributing to As groundwater contamination.

In the fourth chapter of my thesis we evaluate a role of various microbial processes that can affect As groundwater concentration. The fate of As in groundwater can be influenced by multiple microbial and abiotic processes that can lead to its mobilization or immobilization. Yet, most studies to date focused on individual processes, particularly those that lead to As mobilization such as microbially mediated Fe(III) mineral reduction. However, microbially driven As immobilization processes were often overlooked. In this study we combined microbial community analysis with groundwater hydrogeochemical data in order to explain the behavior of As along a 2 km transect across an As-contaminated Holocene aquifer, through the redox transition zone (RTZ) where As immobilization occurs, and further in a pristine Pleistocene aquifer. Our analyses revealed fermentation and methanogenesis as important processes favoring As mobilization. As a consequence of the high CH_4 concentration in the RTZ, anaerobic methanotrophs that can potentially couple CH_4 oxidation to Fe(III), Mn(IV), and SO_4^{2-} reduction, are abundant. Finally, our results underline the role of SO_4^{2-}-reducing and putative Fe(II)-oxidizing bacteria that form poorly soluble minerals as a sink for As. In summary, our results suggest that a complex network of biogeochemical processes has to be considered to fully understand the environmental behavior of As.

Overall, the findings of this PhD thesis bring a new light on the As cycling and highlight the role of microbial processes as important factor affecting fate of As in groundwater. Our study has revealed that not only the quantity, but also the quality and bioavailability of carbon plays a key role in microbially-mediated As mobilization. We consider the work focusing on CH_4-driven As mobilization as particularly important as it reveals a completely new pathway that is relevant for many aquifers similar to this in Vietnam, where As-bearing Fe(III) minerals are abundant and CH_4 is present. Finally, our study highlights that complex biogeochemical interactions that can lead either to a release or an immobilization of As ultimately control its concentration in groundwater.

Zusammenfassung

Arsen (As) ist ein toxisches Metalloid geogenen Ursprungs, das an vielen Orten weltweit zu schwerwiegenden Grundwasserkontaminationen führen kann. Mit Arsen verunreinigtes Wasser kann relativ leicht behandelt werden, weshalb das Vorhandensein von As im Trinkwasser keine Bedrohung für die entwickelten Länder darstellt. Entwicklungsländer, insbesondere in Süd- und Südostasien, die zu den bevölkerungsreichsten Regionen der Welt zählen, sind jedoch besonders betroffen, da der Zugang zu Wasseraufbereitungsanlagen begrenzt ist und viele Menschen, vor allem in ländlichen Gebieten, noch immer auf flache Grundwasserbrunnen angewiesen sind. Darüber hinaus sind viele Grundwasserleiter entlang der wichtigsten Flussdeltas Süd- und Südostasiens, wie z.B. die des Red River, Mekong und Westbengalen, dadurch gekennzeichnet, dass sich reduzierende Bedingungen einstellen, was zu einer reduktiven Auflösung der reichlich vorhandenen As-haltigen Fe(III)-Minerale in den Sedimenten führt. Die Freisetzung von As aus den Sedimenten und die Anreicherung im Grundwasser führte zu dem, was als "die größte Massenvergiftung der Geschichte" beschrieben wurde.

Bis heute wurden verschiedene Mechanismen zur Freisetzung von As in Grundwasser vermutet. Neben der reduktiven Auflösung von Mineralen, sind auch pH-Wert Veränderungen, sowie die Mobilisierung von adsorbiertem As durch Sorptionskonkurrenz mit Phosphat, das durch intensive Düngergaben oder Oxidation arsenhaltiger Sulfidminerale eingebracht wurde, vorgeschlagen worden. Darüber hinaus wurde auch vermutet, dass eine Änderungen der Sorptionskapazität von Fe(III)-(oxyhydr)oxiden zu As-Mobilisierung beitragen kann. Der am häufigsten angenommene Mechanismus ist jedoch, dass As aus Aquifersedimenten während der mikrobiellen reduktiven Auflösung von As-haltigen Fe(III)-(Oxyhydr)oxid-Mineralen freigesetzt wird. Dieser Prozess erfordert jedoch das Vorhandensein von bioverfügbarem Kohlenstoff (C), wie z.B. kurzkettige Fettsäuren, die Fe(III)-reduzierende Bakterien wie *Geobacter* spp. als Energie-, Elektronen- und Kohlenstoffquelle für ihre Aktivität und ihr Wachstum benötigen.

Obwohl sich unser Verständnis der biogeochemischen Prozesse, die zur Mobilität und Verteilung von Arsen in der Umwelt beitragen über die vergangenen zwei Jahrzehnte verbessert hat, bleiben dennoch viele Fragen offen. Insbesondere wenn es um die Rolle biologischer Prozesse und die Beteiligung verschiedener Mikroorganismen geht, die die (Im)mobilität von As im Grundwasser beeinflussen können. Deshalb wurden in dieser Doktorarbeit, mit Blick auf den mit As kontaminierten Aquifer in Van Phuc bei Hanoi (Vietnam), mehrere Experimente

durchgeführt, um zu verstehen, wie verschiedene Arten von organischen Kohlenstoffs die As-Mobilisierung aus Aquifersedimenten beeinflussen kann, welche Rolle CH_4 im As-Kreislauf einnimmt und wie die vorhandene Mikrobengemeinschaft die As-Konzentration im Grundwasser beeinflusst.

Natürliches organisches Material (NOM), als Elektronenquelle für Fe(III)-reduzierende Mikroorganismen, kann indirekt zur Mobilisierung von As aus As-haltigen Fe(III)-(oxyhydr)oxiden beitragen. Die meisten Laborstudien verwendeten jedoch einfache Fettsäuren oder Zucker, oft in relativ hohen Konzentrationen, anstelle von natürlich vorkommendem OM. Wir extrahierten daher in-situ OM aus dem oberen Aquitard (tonhaltiger Schlamm) und den unteren sandigen Aquifer-Sedimenten in Van Phuc (Hanoi/Vietnam), charakterisierten dessen Zusammensetzung und verwendeten Mikrokosmen-Experimente über eine Inkubationsdauer von 100 Tagen mit in-situ Aquifermaterial. Darin wurden die Wirkung von in-situ OM auf die Reduktion von Fe(III)-Mineralien bestimmt, sowie die As Mobilisierung und Zusammensetzung der Mikrobengemeinschaft identifiziert. Wir fanden heraus, dass OM, das aus dem tonigen Schlick-Aquitard (OMC) extrahiert wurde, jungem, nicht vollständig abgebautem, pflanzlichem Material ähnelt, während OM aus tieferen sandigen Sedimenten (OMS) mehr Carboxylgruppen enthält, die sich auf das Vorhandensein von Amino- und Fettsäuren zurückführen lassen. Obwohl alle Mikrokosmen mit der gleichen Menge C (12 mg C/L) angereichert wurden, war das Ausmaß der Fe(III)-Reduktion nach 100 Tagen (43±3,5%) am höchsten in Ansätzen mit Acetat/Laktat, gefolgt von OMS (28±0,3%) und OMC (19±0,8%). Im Gegensatz dazu wurden zwar anfänglich mehr As in den Acetat/Laktat-Ansätzen mobilisiert, doch nach 100 Tagen wurden ähnliche oder sogar höhere Mengen von As (8,3±0,3 und 8,8±0,8 µg As/g Sediment) durch OMC bzw. OMS mobilisiert, verglichen zu Ansätzen mit Laktat/Acetat (6,3±0,7 µg As/g Sediment). 16S rRNA-Amplikonsequenzanalysen zeigten, dass Acetat/Lactat hauptsächlich *Geobacter* spp. anreicherte, während in-situ-OM das Wachstum und die Aktivität einer vielfältigeren Mikrobengemeinschaft unterstützte. Unsere Ergebnisse deuten darauf hin, dass das in-situ OM zwar weniger effizient bei der Stimulation der mikrobiellen Fe(III)-Reduktion ist verglichen mit dem hoch bioverfügbare Acetat/Laktat Gemisch, aber letztlich das Potenzial hat, die gleiche oder sogar mehr As zu mobilisieren.

In vielen mit As kontaminierten Aquiferen wurden hohe Konzentrationen an Methan (CH_4) gemessen, jedoch ist die Rolle von CH_4 für die Mobilisierung von As derzeit völlig unbekannt. In der vorliegenden Studie zeigen wir, dass CH_4 als Elektronenquelle für methanotrophe Mikroorganismen dienen kann und die reduktive Auflösung von As-haltigen Fe(III)-(oxyhydr)oxid-Mineralin auslöst, was zur As-Mobilisierung führt. In Mikrokosmen mit As-

haltigen Sedimenten aus dem Delta des Red River, die mit umweltrelevanten Konzentrationen von CH_4 inkubiert wurden, fanden wir heraus, dass CH_4 die Reduktion von Fe(III)-Mineralen auslöst, das Wachstum und die Aktivität von aeroben Methanotrophen vom Typ 1 und Archaean verstärkt.

Die (Im)mobilität von As im Grundwasser kann durch eine Vielzahl mikrobieller und abiotischer Prozesse beeinflusst werden, die zu seiner Mobilisierung oder Immobilisierung führen können. Die meisten Studien konzentrierten sich bisher jedoch auf einzelne Prozesse, insbesondere auf solche, die zur Mobilisierung von As führen, wie die mikrobiell induzierte Reduktion von As-haltigen Fe(III)-Mineralen. Allerdings wurden mikrobiell bedingte As-Immobilisierungsprozesse oft übersehen. In dieser Studie kombinierten wir die Analyse der mikrobiellen Gemeinschaften mit hydrogeochemischen Grundwasserdaten, um das Verhalten von As entlang eines 2 km langen Transekts durch einen As kontaminierten holozänen Aquifer zu erklären. Darin wurde eine Redox-Übergangszone (RTZ) identifiziert, in der die As Immobilisierung stattfand, die weiter in einen unberührten pleistozänen Aquifer übergeht. Unsere Analysen ergaben, dass Fermentation und Methanogenese wichtige Prozesse sind, die zu einer Reduktion des Redoxpotentials führen, was letztendlich zu einer Mobilisierung von As führen kann. Aufgrund hoher CH_4-Konzentration in der RTZ sind anaerobe Methanotrophe, die die CH_4-Oxidation an die Reduktion von Fe(III), Mn(IV) und SO_4^{2-} koppeln können, reichlich vorhanden. Schließlich unterstreichen unsere Ergebnisse die Rolle von SO_4^{2-}-reduzierenden und mutmaßlich Fe(II)-oxidierenden Bakterien, die schwer lösliche Minerale bilden, die als Senke für As fungieren können. Zusammenfassend legen unsere Ergebnisse nahe, dass ein komplexes Netzwerk biogeochemischer Prozesse innerhalb des Aquifers berücksichtigt werden muss, um das Umweltverhalten von As vollständig zu verstehen.

Insgesamt werfen die Ergebnisse dieser Doktorarbeit ein neues Licht auf den As-Kreislauf und heben die Rolle mikrobieller Prozesse als wichtigen Faktor hervor, der das Schicksal von As im Grundwasser beeinflusst. Unsere Studie hat gezeigt, dass nicht nur die Quantität, sondern auch die Qualität sowie die Bioverfügbarkeit von Kohlenstoff eine Schlüsselrolle bei der mikrobiell induzierten Mobilisierung von As spielen. Wir erachten die Arbeit, die sich auf die CH_4-abhängige As-Mobilisierung konzentriert, als besonders wichtig, da sie einen völlig neuen Weg aufzeigt, der für viele Aquifere ähnlich wie in Vietnam, in denen As-haltige Fe(III)-Minerale im Überfluss vorhanden sind und CH_4 bioverfügbar ist, relevant sein kann. Schließlich hebt unsere Studie hervor, dass komplexe biogeochemische Wechselwirkungen, die entweder zur Freisetzung oder Immobilisierung von As führen können, letztlich die Konzentration von As im Grundwasser kontrollieren.

Chapter 1: Introduction and objectives of this study

Arsenic (As) is the 20th most abundant element in the Earth's crust and it is broadly distributed in the environment (Lenoble et al., 2004). This toxic metalloid is characterized by its intermediate chemical and physical properties between a metal and non-metal (Humans, 2012). In reducing environments, more mobile, more soluble and therefore generally more toxic arsenite (As(III)) is predominant. Arsenate (As(V)) dominates under oxygenated conditions and it is known to be less mobile specie that is more prone to sorption onto minerals and less toxic (Ng et al., 2001; Smedley & Kinniburgh, 2002). Arsenic can form various complexes with other elements, forming both organic and inorganic compounds. On the one hand, among inorganic arsenic trivalent compounds such as arsenic trioxide, sodium arsenite and arsenic trichloride are the most common while arsenic pentoxide, arsenic acid and arsenates are the most common pentavalent compounds. On the other hand, prevalent organic arsenic compounds include arsanilic acid, methylarsonic acid, dimethylarsinic acid, and arsenobetaine (Humans, 2012; WHO, 2001). There are many potential sources of As in the environment. It can be emitted as result of volcanic or industrial activities such as mining, smelting of non-ferrous metals and burning of fossil fuels leading to contamination of air, water, and soil (mainly as arsenic trioxide). Moreover, arsenic-containing pesticides, commonly used in the past, have left large amount of As in agricultural lands until these days (Peryea, 1991). Furthermore, As is ubiquitous in many rocks being a main constituent of over 200 minerals, of which 60% are arsenates, 20% sulfides and sulfosalts, whereas the remaining 20% include arsenides, arsenites, oxides, silicates and elemental As (Thornton, 1996).

1.1 Arsenic exposure and its health consequences

Exposure to As and its compounds is considered as a major public health concern due to its toxic effect on human health and its clear carcinogenic potential (Water et al., 1999). The World Health Organization (WHO) estimated that over 140 million people worldwide might be chronically exposed to As in the drinking water at a concentration overpassing WHO safety standards of 10 µg/L (WHO, 2008). Arsenic holds the highest ranking on the current U.S. Agency for Toxic Substances and Disease Registry (ATSDR) priority list and due to its toxicity, the International Agency for Research on Cancer (IARC) defines arsenic as a Group I human carcinogen (Naujokas et al., 2013). Although As contaminates mainly surface water and groundwater, ultimately it enters into the food chain as the water is used for cooking and irrigation, leading to its accumulation in vegetables, rice and fish (Arain et al., 2009; Molin et

al., 2015; Muhammad et al., 2010). Because As is “invisible” (it does not affect the taste or smell of the water and food), many people have been unconsciously consuming it over years. Arsenical skin lesions are the first hallmark of chronic As poisoning (Fatmi et al., 2009; Karagas et al., 2015). Ingestion of As-contaminated water and food over prolonged time subsequently can lead to more severe health consequences including, restrictive lung disease, cardiovascular and central nervous system disease, and increased risk of various forms of cancer (Chen et al., 2011; Smith A H et al., 1992). South and Southeast Asia, which is also the most densely populated part of the World (Smedley & Kinniburgh, 2002; Smith et al., 2000) is particularly prone to this problem due to insufficient access to water treatment facilities, which are available mainly to bigger cities. Inhabitants of most rural areas still rely on shallow groundwater wells for drinking water supply, that is often treated with simple sand filters that are often not maintained appropriately to fulfill its function (Nitzsche et al., 2015). As a consequence, more than 20% of all deaths in highly affected areas of Bangladesh were linked to As poisoning (Ravenscroft P et al., 2009). Although awareness regarding potential risks associated with consumption of untreated water has increased substantially over the last years, many people from rural areas simply have no access to safe water or cannot afford to purchase commercially available filters. Moreover, while household sand filters might be very efficient to remove As from Fe-rich water such as in Vietnam, natural Fe concentrations in Bangladesh’s waters are usually too low for passive removal or simple sand filtration (Leupin et al., 2005).

1.2 Source of As in South and Southeast Asia

It is widely accepted that As in South and Southeast Asia is mainly of geogenic origins. Erosion and weathering of metamorphic rocks of the Himalayan Mountains released sediments that were washed down the slopes, transported with rivers and deposited along the major rivers and river deltas of South and Southeast Asia (McArthur et al., 2001; Mukherjee et al., 2009; Polizzotto et al., 2008). During this geological process, iron-loaded rocks eroded into iron (oxyhydr)oxides-coated silty and sandy sediments with high affinities for As. Particles coated with Fe and adsorbed As were washed and transported downstream (Figure 1.1). Therefore, As was brought to the river deltas, sorbed to solids and deposited in the soil with the settling sediments. Suspended solid particles were preferentially deposited in flat floodplains of the Red River, Mekong, West Bengal, Ganges and Indus Deltas. For thousands of years, the process of sediment deposition, that was particularly pronounced in the Holocene period (last 10,000–12,000 years) (Tanabe et al., 2006), created the soil layers (sediments) that formed the deltas, as we know them today. These sediments reach more than a hundred meters below today's

topsoil layer burying in their composition As and crates what are now aquifers. Although a large amount of As was deposited over geological time, it appears that fresh river deposits replenish new supplies of As (Wallis et al. 2020).

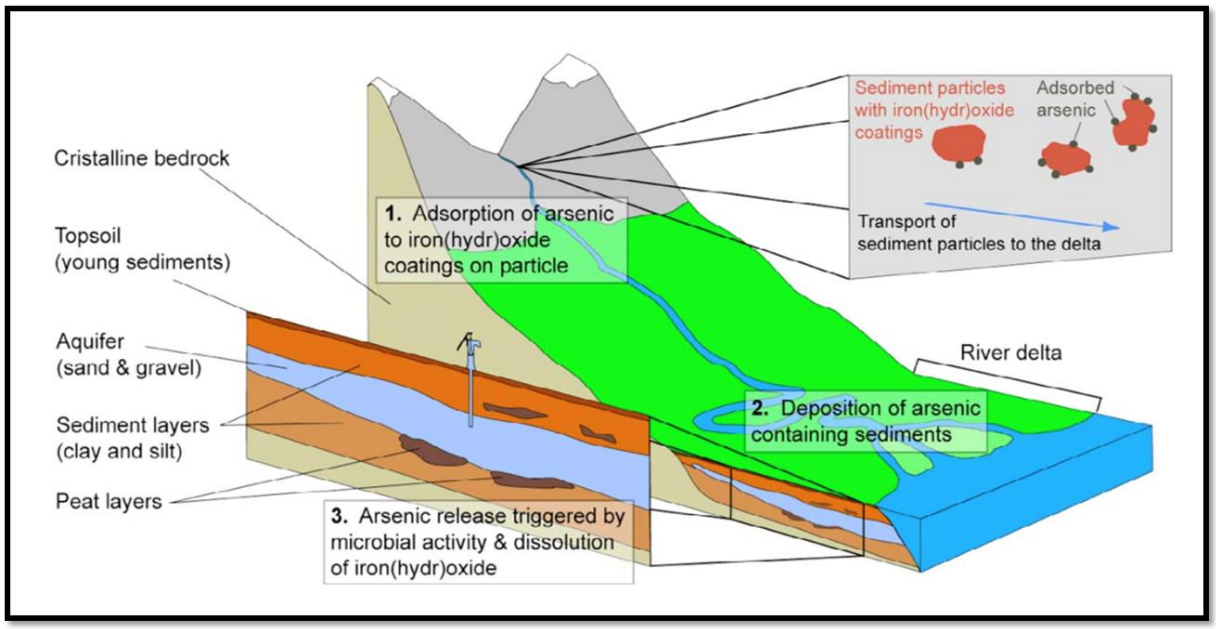

Figure 1.1 Deposition of Fe and As containing sediments along river deltas of South and Southeast Asia. Source: Berg, 2007

1.3 Mechanisms of As mobilization to groundwater

Release of geogenic As from sediments to groundwater can happen through several different mechanisms. Although many pathways of As mobilization have been proposed to date, in reality this process strongly depends on local hydrology, geology, geochemistry as well as anthropogenic activities (Bhattacharya et al., 2010) and the microbial community mediating biogeochemical processes. Moreover, rates and extent of As mobilization likely depend on a combination of these interacting factors rather than the effect of a single process. The intensive groundwater abstraction and irrigation – leading to oxic conditions by decreasing water levels – and the oxidation of As-rich sulfides (mainly pyrites) present in aquifer sediments has been proposed as one possible pathway (Chowdhury et al., 1999; Das et al., 1996; Mandal et al., 1996). This mechanism, however, was largely dismissed, particularly in the Bengal Basin and other similar aquifers where As-bearing pyrite was found only sporadically at rather low abundance which could not release the significant amount of dissolved As (Acharyya et al., 2000; Umitsu, 1993). Additional evidence comes from the observation that only very low concentrations of dissolved sulfate were detected, which is produced during pyrite oxidation, (Acharyya et al., 2005) and strongly reducing conditions in As-contaminated aquifers (von Brömssen et al., 2007) that would suppress oxidative processes.

Another proposed mechanism suggested that As is mobilized as a result of competitive sorption and exchange of As with phosphorus (P) supplied with fertilizers onto Fe(III) mineral surfaces (Chowdhury et al., 1999). However, the quantity of P needed to mobilize As concentrations observed in the field is unrealistic considering the depths of the aquifers and low mobility of P (Bose & Sharma, 2002) as well as the low permeability of aquitards efficiently preventing the percolation of water. A similar mechanism of competitive sorption between As and carbonates (Appelo et al., 2002) was also proposed. Changes in sorption capacities of Fe(III) (oxyhdr)oxides triggered by the development of a high pH (>8.5) due to mineral weathering and high evaporation rates were also shown to mobilize As. The increased pH can either lead to the desorption of adsorbed As, or prevent it from being adsorbed to Fe(III) minerals (Smedley & Kinniburgh, 2002).

One of the prevailing theories for As mobilization is through reductive dissolution of As-rich Fe(III) (oxyhydr)oxides under anoxic conditions (Harvey et al., 2002; Nickson et al., 1998; Smedley & Kinniburgh, 2002). Presence of buried organic matter (often referred to as peat lenses) at different depths of aquifers promotes strongly reducing conditions due to biodegradation and fermentation of organic carbon (Postma et al., 2007; Quicksall et al., 2008). Moreover, slow or completely limited diffusion of O_2 through sediment layers, particularly through clay silt aquitards, effectively prevents the entrance of O_2 to aquifers further decreasing their redox potential. This encourages the reduction of As(V) and desorption from Fe(III) (oxyhydr)oxides, as well as the reductive dissolution of Fe(III) minerals (Datta, 2015). Additionally, organic matter is known to create complexes with As and Fe (Sharma et al., 2010) leading to a significant accumulation of As within peat lenses, thus representing a potential source of As (Shotyk et al., 1996). Therefore, biodegradation of organic matter may not only initiate reducing conditions by decreasing the local redox potential, but it can directly release As from OM-Fe-As complexes by reductive dissolution.

Undoubtedly, a combination of abiotic and biotic processes described above may contribute to As mobilization, yet it is commonly accepted that microorganism play a decisive role in catalyzing reductive dissolution of As bearing Fe(III) minerals present in aquifer sediments and ultimately releasing of As to groundwater (Farhana S. Islam et al., 2004). It is clear that microbially mediated reduction of Fe(III) (oxyhydr)oxides is coupled to C oxidation (Chatain et al., 2005; Héry, M. et al., 2010; F. S. Islam, Pederick, et al., 2005; Neumann et al., 2014) and various microorganisms such as *Geobacter* spp. (F. S. Islam, Pederick, et al., 2005), *Shewanella* spp. (Cummings et al., 1999) and *Geothrix* spp. (F. S. Islam, Boothman, et al., 2005) can mediate this reaction. This process, however, requires sufficient quantities of bioavailable

organic C, which might be a limiting factor for As mobilization in some aquifers. While past studies have probed the effects of different quantities and identities of C sources in As mobilization (Duan et al., 2008; Lawson et al., 2013; Mailloux et al., 2013; Solaiman et al., 2009), those studies tend to use easily bioavailable short-chain fatty acids which does not reflect natural conditions, potentially leading to an overestimation of the amount of As released.

These are some of the mechanisms that received the most attention. Yet, there is an increasing number of studies that show new unexplored pathways for As remobilization as well as immobilization that will be further discussed in Chapters 3, 4 and 5.

1.4 Field site description and sampling campaigns

The study site, Van Phuc village, is located inside the Red River meander, about 15 km Southeast from Hanoi (20°55'18.7"N, 105°53'37.9"E) (Figure 1.2).

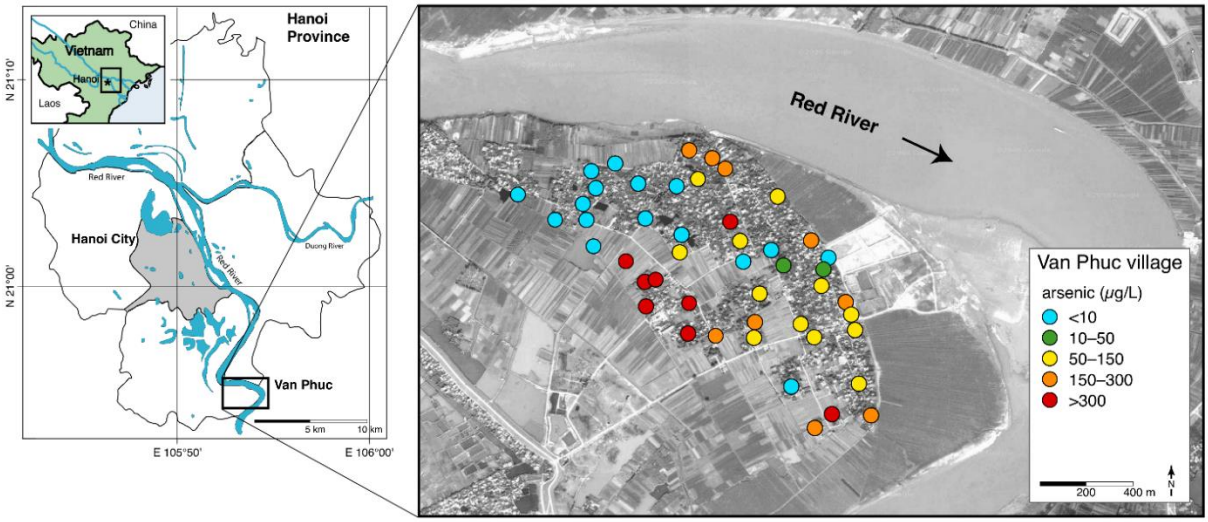

Figure 1.2 Left: map of Hanoi province with the zoom to Van Phuc village (right). The map of Van Phuc demonstrates the distribution of monitoring wells and As groundwater concentrations. Source: modified based on Eiche et al., 2017

Studies investigating the As contamination in and around Van Phuc have been conducted for over two decades. Therefore, a substantial number of publications is available that describe in detail the mineralogy, hydrology, lithology and geology of the Van Phuc aquifer (Berg et al., 2008; Eiche et al., 2008, 2017; Stopelli et al., 2020; van Geen et al., 2013). Briefly, due to a number of sedimentation processes, the lithology of this deltaic sediments in Van Phuc vary considerably within short distances (Mathers & Zalasiewicz, 1999; Tanabe et al., 2006). The North-Western area is characterized by orange sands dating back to Pleistocene and groundwater with As concentrations below the WHO guideline (10 µg/L). In contrast, the aquifer of the South-Eastern part is compiled of (young) Holocene gray sediments where As in

the groundwater exceeds the 10 µg/L limit by a factor of 10-50 (Eiche et al., 2008). Although sedimentary As concentrations in orange and gray sediments is rather similar (~5 mg/kg on average), in the Holocene sediments As is mainly associated with mixed valence Fe(II/III) minerals, while in Pleistocene sands As is not adsorbed but more tightly bound mostly to orange-brown Fe(III) oxides (Eiche et al., 2008) (Figure 1.3).

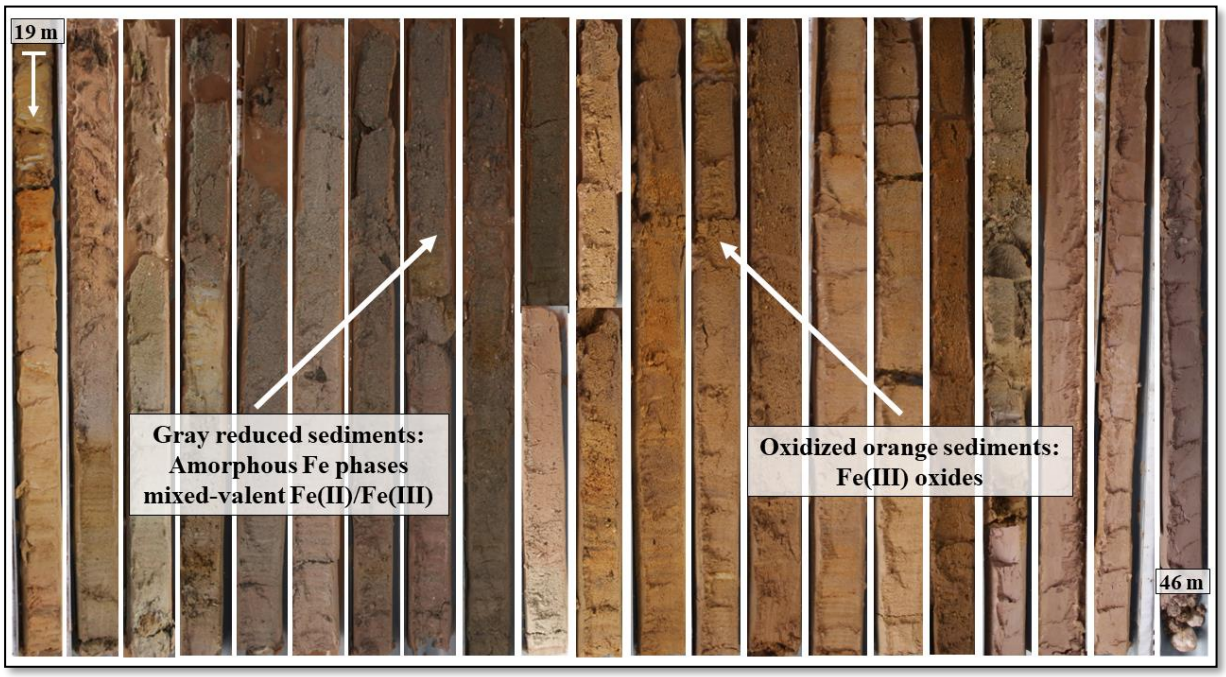

Figure 1.3. Sediment cores collected during 2017 sampling campaign. Each core was 1 m long and 10 cm diameter. Changes in redox conditions are distinguishable by sediments' color.

The transition between the contaminated and uncontaminated zones is characterized by changing redox conditions. The redox transition zone at the boundary of the two aquifers is where a change from highly to moderately reducing conditions was observed and where As rich water from the Holocene aquifer migrates into the Pleistocene aquifer (Stopelli et al., 2020; van Geen et al., 2013). However, As penetration into the uncontaminated Pleistocene aquifer is retarded due to enhanced sorption of As onto Fe(III) (oxyhydr)oxides, which have a high surface area and are more abundant in Pleistocene sands (Rathi et al., 2017). But also authigenic minerals produced by redox changes in the transition zone (Rawson et al., 2016) and other potential reactions efficiently prevent the spreading of As contaminated water into pristine (As below 10µg/L) aquifers. The Pleistocene and Holocene aquifers are mainly recharged by water from the river. An extensive groundwater abstraction in Hanoi city caused a depression cone and reversal of the natural groundwater flow direction that flow towards the capital with an estimated velocity of 40 m/year (van Geen et al., 2013). Generally, the aquifer consists of organic-rich clayey silt aquitard of variable thickness overlying loose bedding of gray Holocene and orange Pleistocene sandy sediments (both containing OM inclusions) reaching over 40 m

depth (Berg et al., 2008; Eiche et al., 2017). The fertile soils in Van Phuc area consisting of organic river muds are mainly used for agricultural purposes (cultivation of maize, bananas, citrus, vegetables) that are supplied with NPK fertilizers often in uncontrolled way. In Van Phuc and its surrounding, numerous ponds often used for fish and duck production, are common as well as dense network of irrigation channels and small water reservoirs. Until recently, groundwater was the main source of drinking water in Van Phuc and household sand filters have been efficiently used to remove Fe and As (Berg et al., 2006; Nitzsche et al., 2015).

Water abstraction from low-As Pleistocene aquifers became a main mitigation strategy. This, however, disturbed groundwater flow and can potentially draw high-As groundwater into to low-As aquifers. In fact, due to the constantly increasing water demand and groundwater pumping, currently pristine aquifers are at high risk of becoming As-contaminated (Berg et al., 2008; van Geen et al., 2013; Winkel et al., 2011). Understanding As dynamic in aquifers is of great importance in order to prevent new cases of As exposure and reduce a health threat of local communities. To investigate As dissolution, advection and retardation across redox gradients and ultimately predict the large-scale and long-term mobility of As under enhanced hydraulic forcing a multidisciplinary collaboration was established. Thus, this PhD thesis is a part of a joint international project entitled “Retardation and mobilization of arsenic at redox fronts under advective flow conditions - a concerted multidisciplinary approach (AdvectAs)”. Further information about the AdvectAs project is provided in Statement of Personal Contribution.

Two sampling campaigns took place in Van Phuc to collect samples necessary for this PhD project. In October 2017, the first sampling campaign was performed. During this campaign, rotary drilling (Figure 1.4) was carried out to collect sediment samples as well as groundwater samples from several monitoring wells within Van Phuc village. The overarching goals of this first field campaign were the collection of aquifer sediments to conduct microcosms experiments, to obtain preliminary information about the abundance and distribution of Fe(III)-reducing bacteria, and to characterize microbial communities in selected samples across the vertical profile of the aquifer. Groundwater samples were collected from selected wells to gather preliminary information about microbial communities’ composition. Data on groundwater hydrogeochemistry, solid phase chemistry, mineralogy and dissolved gases were collected by project collaborators. Based on the results of the first sampling campaign, a new hypothesis regarding role of CH_4 in As mobilization was formulated. In order to validate this new hypothesis, sediment samples were collected during the second sampling campaign that took place in November 2018. During the 2018 sampling campaign, another rotary drilling was

performed (3 m away from the 2017 drilling location). Aquifer sediment samples were collected in order to set up another microcosm experiment. Furthermore, all available monitoring wells were sampled and biomass was collected for microbial community analysis across the transect.

Figure 1.4 Location of drilling site and images from 2017 sampling campaign including sediment cores and groundwater collection. Picture courtesy of Prof. Dr. A. Kappler and Monique Patzner.

1.5 Objectives of this study

Although several mechanisms for a mobilization of As from aquifer sediments have been proposed (Subchapter 1.3), most of them focused on abiotic processes. Except for microbially mediated Fe(III) reduction, not much of attention has been given to biotic processes that can affect the fate of As in groundwater. Moreover, many studies were conducted to identify microorganisms involved in Fe(III) reduction and to estimate how much As can be mobilized. To date, the role of organic matter which is essential to fuel this process, was not investigated in detail. Therefore, in order to improve our knowledge and understanding on how microorganism can influence the behavior of As in groundwater aquifers, the objectives of this study are:

- To evaluate the importance and properties of natural organic matter (NOM) fueling microbially mediated reductive dissolution of As-bearing Fe(III) minerals present in aquifer sediments and to appraise its potential to mobilize As compared to commonly used and easily bioavailable organic carbon (C) sources such as acetate and lactate (**Chapter 2**).
- To demonstrate the important role of methane (CH_4) that is abundantly present in Van Phuc but also in other similar As-contaminated aquifers in South and Southeast Asia, as electron donor for microbially mediated Fe(III) reduction. Until today CH_4 was not considered as important C source and has never been linked to As mobilization in aquifers (**Chapter 3**).
- To assess function and influence of various microbial groups present in groundwater across different geochemical zones on As (im)mobilization. To date, most studies concentrated on Fe(III)-reducing microorganism, although, known Fe(III)-reducers are not very abundant in aquifers. At the same time, other groups of microorganisms such as fermenters, methanotrophs, methanogens or sulfate-reducing bacteria dominate in As-contaminated aquifers such as Van Phuc. Yet, they were largely overlooked and their role in As cycling is not fully understood (**Chapter 4**).
- To propose new and unexplored mechanisms of As mobilization and immobilization, evaluate their importance, potential occurrence and contribution to As behavior. This chapter aim to summarize the role of multiple biological processes that importantly influence As in groundwater and overall water quality (**Chapter 5**).

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Chapter 2 – Personal contribution

The sampling campaigned in Vietnam was organized by Prof. Michael Berg and jointly performed by all AdvectAs project members. Sediment samples for the microcosm experiment were collected by myself. The hypothesis was formulated by myself and Prof. Dr. Andreas Kappler. Experiments were conceptualized, setup and conducted by myself. The data collection was carried out by myself. The ICP-MS analysis was performed by Dr. Emiliano Stopelli. The organic matter analysis was performed by Dr. Heike Knicker (^{13}C-NMR) and Dr. Julie Tolu (Pyrolysis-GC/MS). The discussion and analysis of the obtained results were done by myself and Prof. A. Kappler. The manuscript was written by myself with the support of Prof. A. Kappler and revised by all co-authors.

Chapter 2: Role of in-situ natural organic matter in mobilizing As during microbial reduction of Fe^{III}-mineral-bearing aquifer sediments from Hanoi (Vietnam)

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2.1 Abstract

Natural organic matter (NOM) can contribute to arsenic (As) mobilization as an electron donor for microbially-mediated reductive dissolution of As-bearing Fe(III) (oxyhydr)oxides. However, to investigate this process, instead of using NOM, most laboratory studies used simple fatty acids or sugars, often at relatively high concentrations. To investigate the role of relevant C sources, we therefore extracted *in-situ* NOM from the upper aquitard (clayey silt) and lower sandy aquifer sediments in Van Phuc (Hanoi area, Vietnam), characterized its composition, and used 100-day microcosm experiments to determine the effect of *in-situ* OM on Fe(III) mineral reduction, As mobilization and microbial community composition. We found that OM extracted from the clayey silt (OMC) aquitard resembles young, not fully degraded plant-related material, while OM from the sandy sediments (OMS) is more bioavailable and related to microbial biomass. Although all microcosms were amended with the same amount of C (12 mg C/L), the extent of Fe(III) reduction after 100 days was highest with acetate/lactate (43±3.5% of total Fe present in the sediments) followed by OMS (28±0.3%) and OMC (19±0.8%). Initial Fe(III) reduction rates were also higher with acetate/lactate (0.53 mg Fe(II) in 6 days) than with OMS and OMC (0.18 and 0.08 mg Fe(II) in 6 days, respectively). Although initially more dissolved As was detected in the acetate/lactate setups, after 100 days, higher concentrations of As (8.3±0.3 and 8.8±0.8 µg As/L) were reached in OMC and OMS, respectively, compared to lactate/acetate-amended setups (6.3±0.7 µg As/L). 16S rRNA amplicon sequence analyses revealed that acetate/lactate mainly enriched *Geobacter* while *in-situ* OM supported growth and activity of a more diverse microbial community. Our results suggest that although the *in-situ* NOM is less efficient in stimulating microbial Fe(III) reduction than highly bioavailable acetate/lactate, it ultimately has the potential to mobilize the same amount or even more As.

2.2 Introduction

Arsenic (As) is a toxic metalloid that causes serious health issues such as arsenicosis, cardiovascular disease and increased risk of cancer (Chen et al., 2011; Smith A H et al., 1992). It is estimated that over 140 million people from 50 countries are at risk of consuming water with As concentrations exceeding the recommended limit of 10 µg/L (Ravenscroft P et al., 2009). Southeast Asia is a particularly affected part of the world (Berg et al., 2001). Due to insufficient access to central water supplies and water treatment facilities, many people still rely on shallow groundwater wells. As a consequence, more than 20% of all deaths in highly affected areas of Bangladesh were linked to As poisoning (Ravenscroft P et al., 2009). Although

our knowledge about processes affecting As mobilization has increased substantially in recent years (Muehe & Kappler, 2014; Zhu et al., 2017), many questions still remain regarding the identity and mechanisms of microbial and abiotic processes responsible for As release from As-bearing minerals.

It is generally accepted that the mobilization of As from the aquifer sediments into groundwater is mainly due to microbially-mediated reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals (Lear et al., 2007; Polizzotto et al., 2006; Sutton et al., 2009; A. van Geen et al., 2004). Organic matter (OM) plays a key role in this process, in particular as electron donor for microorganisms (Akai et al., 2004; H. M. Anawar et al., 2006; F. S. Islam et al., 2005; Lapworth et al., 2008). It has been demonstrated both in microcosms as well as in *in-situ* experiments that high concentrations (5-50 mM) of easily bioavailable carbon sources such as acetate, lactate, glucose, polypepton or urea stimulate microbial activity and trigger the reductive dissolution of Fe(III) minerals, with the subsequent mobilization of As that was associated with the minerals (Akai et al., 2004; Duan et al., 2008; Gault et al., 2016; Neidhardt et al., 2014; Radloff et al., 2007, 2007; Rowland et al., 2007). However, only a few studies have investigated the effect of environmentally relevant organic C (e.g. DOC-rich water from paddy soil or ponds) on As mobilization without amendment of labile C (Akai et al., 2004; Neumann et al., 2014). Additional organic compounds that have been tested in such studies are humic substances or water from a drainage tube (Bauer & Blodau, 2006) and plant material such as ground bean leaves, barley straw or pine sawdust (Solaiman et al., 2009). Such carbon sources, however, are mostly relevant for shallow aquifers where potential leaching or percolation from the surface could happen, and not for the OM that is present in deeper aquifer layers. To our knowledge, no studies have explicitly extracted naturally occurring (*in-situ*) OM from sediments and used it in sediment microcosms. There is still a lack of reliable, quantitative Fe(III) reduction and As mobilization data with environmentally relevant sources and concentrations of carbon. Furthermore, there is no detailed information about microbial taxa directly involved in the Fe(III) mineral reduction processes using this *in-situ* C as electron donor.

The OM present in As-contaminated aquifers can have different origins. It can be introduced from anthropogenic (wastewater, fertilizers, oil spills) or natural sources (rivers, ponds) through water recharge from the surface or liberated from the sediments (e.g. previously buried peat layers) (Akai et al., 2004; Ghosh et al., 2015; McArthur et al., 2004). These C sources can contain complex plant-based OM which is considered rather resistant to chemical and biological degradation (Marschner et al., 2008; Ruiz-Dueñas & Martínez, 2009) as well as labile

low-molecular-weight C such as amino acids, carbohydrates and carboxylic acids that can be easily used by microorganisms to fuel microbially mediated Fe(III) reduction leading to As release (Hossain Md. Anawar et al., 2013; Berggren et al., 2010; Rowland et al., 2007). Therefore, the identity and bioavailability of the C present in the aquifer is key to understanding its potential role in As mobilization.

For the present study, we chose an aquifer in the village of Van Phuc, about 15 km SE of Hanoi, which shows a large variability in dissolved As concentrations (van Geen et al., 2013). An organic-rich clayey silt aquitard of variable thickness overlies loose beddings of grey Holocene and orange Pleistocene sandy sediments (both containing OM inclusions) reaching over 40 m depth (Berg et al., 2008; Alexander van Geen et al., 2013; Weinman, 2010). The dominating type of C present in the aquitard and aquifer was derived from vascular C_3 vegetation, freshwater and marine C such as phytoplankton, terrestrial plants and algae (Eiche et al., 2017). It is unknown, however, to which extent this OM can be utilized by microorganisms for Fe(III) reduction and As mobilization. Therefore, we chose a novel approach of using extracted *in-situ* NOM as a source of C in our incubation experiment. We extracted and characterized OM from the clayey/silty and sandy sediments in Van Phuc. We then used this OM in batch microcosms to assess the rates and the extent of Fe(III) reduction and As release in comparison to microcosms with commonly used easily bioavailable C sources (acetate/lactate). Finally, we identified the microorganisms mediating these processes over the course of the experiment.

2.3 Materials and Methods

2.3.1 Study area and sample collection

The sampling site is situated close to Van Phuc village, about 15 km SE from Hanoi, inside a meander of the Red River (20°55'18.7"N, 105°53'37.9"E). The lithology, mineralogy, geology and information about OM composition and distribution were described previously (Eiche et al., 2008, 2017; Alexander van Geen et al., 2013; Weinman, 2010). Briefly, the North-Western area is characterized by Pleistocene aquifer sands and groundwater with As concentrations below the WHO guideline (10 µg/L), whereas the aquifer of the South-Eastern part is of (young) Holocene age where groundwater exceeds the 10 µg/L limit by a factor of 10-50 (Eiche et al., 2008). The transition between the contaminated and uncontaminated zones is characterized by changing redox conditions. In October 2017, we collected a sediment core (ø10 cm; each individual piece ca. 3 m long) up to 46 m below ground level at this redox transition zone using rotary drilling. For OM extraction, clayey silt organic-rich aquitard sediments from 11 m depth

that contained some plant residues and orange sandy organic-poor sediments with dark patches from 21 m depth were used (the OM extracted from these layers is termed OMC and OMS). We chose these sediments for OM extractions because they were expected to release OM fueling microbial Fe(III) mineral reduction. For the microcosm setups we chose the orange sediments from 30 m depth because our preliminary data showed that they had high As and Fe contents, they were the most homogenous regarding lithology and color (which allowed to obtain enough representative material for all microcosms), and these sediments are expected to be responsible for the As release observed at that field site. All sediments were stored anoxically at 4°C in the dark until use (3 months). In order to evaluate whether acetate and lactate were present in the aquifer, pore water from sandy sediments was collected by centrifugation and subjected to volatile fatty acid (VFA) analyses with a detection limit of 0.2 µM, as described previously (Laufer et al., 2016). The total Fe and As contents of the 30-m sediment were determined by XRF (Bruker, AXS S4 Explorer).

2.3.2 Organic Matter Extraction and Characterization

The dominating type of C present in the aquifer originates from vascular C3 plants (mainly mangroves) (Eiche et al., 2017). Percolation of organic-rich anthropogenic water from the surface is efficiently reduced due to an up to 20 m thick clayey silt layer with low permeability. In order to obtain the potentially bioavailable OM, i.e. the mobile fraction of OM, water extraction was applied. For OM extraction, 100 g of sediments were mixed with 1 L anoxic MilliQ water (bubbled with N_2 for 60 min), shaken (72 h, 20 rpm) in the dark, and centrifuged (30 min; 10,000 rpm). The supernatant was filtered (0.22 µm, PES, Merck™ Steritop™, Millipore). The filtrate was collected and freeze-dried. Samples of the freeze-dried material and bulk sediments from which OM was extracted were used for total organic carbon (TOC) analysis, Fourier-Transform Infrared Spectroscopy (FTIR), ^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR), Excitation-Emission Matrix (EEM) fluorescence spectroscopy, and Pyrolysis Gas Chromatography/Mass Spectrometry (Pyrolysis-GC/MS) analyses as described in Tolu et al. (see SI) (Tolu et al., 2015). The freeze-dried material was re-dissolved completely (no particles remaining) in sterile anoxic MilliQ water. Microwave Plasma-Atomic Emission Spectrometer (MP-AES) analysis (4200, Agilent Technologies, USA) of the solutions was used to quantify the inorganic ions present in the extracted material (Table S2.1) and the DOC of these solutions was quantified by a DOC analyzer (highTOC; Elementar, Germany). 15 mM C stock solutions were prepared and used for preparation of the medium for the microcosms.

2.3.3 Microcosm Setup

Sacrificial microcosms were set up by mixing 1 g of sediment from 30 m depth (orange sandy Fe- and As-bearing sediments that were suggested to be susceptible to As mobilization when exposed to mobile carbon (Fendorf S et al., 2010)) with 5 mL (final volume) sterile synthetic groundwater medium supplemented with C (modified from Rathi et al., 2017); without As and Fe in the medium) in glass vials (total volume 20 mL). Prior to the preparation of the microcosms, the pH of the medium was adjusted to a pH of 7.2 by bubbling with CO_2. The pH was monitored along the experiment and it stayed in the range of 7.2-7.5. Five different C treatments (all containing sediment) were prepared (see Table S2.2): 1) biotic control (CON+), no amendments; 2) abiotic control (CON-), amended with 160 mM sodium azide (NaN_3) and 1 mM carbon (12 mg C/L) as acetate/lactate mix (half of the C from acetate, half from lactate); 3) amended with 1 mM carbon as OMC; 4) amended with 1 mM carbon as OMS; 5) amended with 1 mM C as acetate/lactate mix. It has to be noted that the amount of carbon added was three times the amount of carbon (DOC) that was determined in the groundwater of the drilling site (E. Stopelli, unpublished data). All microcosms were prepared in an anoxic glovebox (100% N_2), closed with rubber stoppers and aluminum caps and flushed with N_2/CO_2 (9/1) in order to maintain anoxic conditions. Afterwards microcosms were kept at 28°C in the dark until analysis (without shaking). At each time point (day 0, 2, 6, 10, 23, 44, 63, 80, 100) 3 vials of each treatment were sacrificed for geochemical analysis and analyzed in triplicate. Six vials were collected for molecular studies at 3 time points (day 0, 10 and 100).

2.3.4 Geochemical Analysis

Vials collected for geochemical analyses were centrifuged at 4000 rpm for 10 min. 100 µL of the supernatant were stabilized in 1M HCl (to avoid oxidation of Fe(II)) and diluted with HCl if necessary for dissolved Fe^{2+} quantification using the Ferrozine assay (depending on the Fe concentration the samples were diluted either in 400 or 900 µL of 1M HCl resulting in a final HCl concentration of 0.2 or 0.1M) (Schaedler et al., 2018). One mL of the supernatant was filtered (0.22 µm) and stabilized in 1% HNO_3 for As analysis by ICP-MS (8900, Agilent Technologies, USA). The remaining liquid phase was used for HPLC quantification of lactate and acetate (Dippon, U. et al., 2017). One g of sediment (wet weight) obtained after centrifugation was digested for 1 h with 2 ml of 6M HCl. 2 mL of the digests were centrifuged (5 min, 14000 rpm) and 100 µL of the supernatant was diluted in 1M HCl. Fe(II) was quantified in triplicate using the Ferrozine Assay (Schaedler et al., 2018). Differences in As and Fe concentration in the different microcosm setups were analyzed with single factor ANOVA and statistical differences in Fe and As at selected time points between pairs of treatments were

determined using the Student's *t*-test. The PhreeqC v3 and minteq.v4 database were used in order to calculate saturation indices (SI) and potential Fe(II) mineral formation at given time points based on the available geochemical data.

2.3.5 Microbial Community Analysis and quantitative PCR

Samples were collected at the beginning of the experiment, after 10 days (when maximum Fe(III) reduction and As release were observed) and at the end of the experiment (100 days). DNA extraction was performed following a protocol from Lueders et al. (Lueders et al., 2004). Bacterial and archaeal 16S rRNA genes were amplified using universal primers 515f: GTGYCAGCMGCCGCGGTAA (Parada et al., 2016) and 806r: GGACTACNVGGGTWTCTAAT (Apprill et al., 2015) fused to Illumina adapters. Subsequent library preparation steps (Nextera, Illumina) and 250 bp paired-end sequencing with MiSeq (Illumina, San Diego, CA, USA) using v2 chemistry were performed by Microsynth AG (Switzerland) and between 49,000 and 75,000 read pairs were obtained for each sample. Sequence analysis was performed as described in the SI. Raw sequencing data can be found at the NCBI Sequence Read Archive (SRA); accession number PRJNA542106 (https://www.ncbi.nlm.nih.gov/sra/PRJNA542106).

Quantitative PCR (qPCR) specific for the 16S rRNA (genes) of bacteria and archaea as well as for arsenate reductase (*arr*A) and anaerobic arsenite oxidase (*arx*A) genes were performed using an iQ5 real-time PCR system (iQ5 optical system software, version 2.0, Bio-Rad). qPCR primer sequences, gene-specific plasmid standards, and details of the thermal programs are given in the SI (Table S2.3).

2.4 Results and Discussion

2.4.1 Identity and characterization of extracted organic matter

TOC analysis of the 45 m drilling core showed that the Van Phuc aquitard contains organic-rich clayey silt whereas the aquifer consists of rather organic-poor sandy sediments with heterogeneously distributed organic inclusions (Eiche et al., 2008, 2017). Sediments at 11 and 21 m depth were selected for OM extraction as representative samples for the organic matter intercalations within the clayey silt aquitard and the sandy aquifers (Figure S2.1). The TOC of the clayey silt material was 9.5±0.15 wt% whereas the sandy sediment contained 0.04±0.0014 wt% of TOC.

The two extracted OM fractions were analyzed by FTIR, ^{13}C-NMR and fluorescence spectroscopy (excitation-emission matrices, EEM). The FTIR spectra of both, OM from clay

(OMC) and OM from sand (OMS) (Figure 2.1A) were generally similar to each other (a certain similarity between OMC and OMS was also confirmed by similar EEM spectra, Figure S2.2), with a few specific differences. In both OMC and OMS, we identified prominent FTIR peaks between 1300 and 900 cm^{-1}, corresponding to the stretching modes of alcoholic C-O, ether C-O-C or O-H deformation (Coates, 2006), characteristic for polysaccharides. The peak at 1616 cm^{-1}, specific for aromatic C=C (alkene) and conjugated C=O or C=N (Coates, 2006), was more pronounced in the OMC spectrum, suggesting the presence of lignin-derivatives (Boeriu et al., 2004) or other aromatics that are also present but less abundant in the OMS. Furthermore, OMC showed a stronger absorption between 3750-3000 cm^{-1}. This region is typical for OH stretching modes that can be related to plant-based molecules such as cellulose as well as for N-H bonds of amines, including amino acids (Coates, 2006). In OMS, a sharp carboxylic peak (COO^-) appeared at 1383 cm^{-1}, most likely related to the presence of amino and fatty acids, pointing towards microbially related C (Kelly & Scheibling, 2012).

In addition to FTIR, solid-state ^{13}C-NMR was applied to characterize the chemical properties of both types of extracted OM (Figure 2.1B). Overall, ^{13}C-NMR analysis also showed a similar presence of the main carbon functional groups in OMC and OMS with alkyl C and O-alkyl C (stemming from carbohydrates) being the most abundant C-functional groups in both extracted OMs. The N-alkyl C as well as the aryl C, which indicate aromatic compounds and phenols (e.g. lignin or lignin degradation products) (Schöning et al., 2005), were also present in both OMC and OMS. Bulk sediments from which OMC and OMS were extracted, were also analyzed by NMR and FTIR in order to evaluate whether the extracted OMs were representative for the sedimentary OM. Although the abundance of some functional groups changed as a result of the extraction process (^{13}C-NMR spectra; Figure S2.3), generally, the NMR intensity distribution of different C-functional groups in both extracted OMs and in the two bulk sediments (Figure 2.1B) showed similar patterns. FTIR spectra of the bulk sediments compared to the spectra of the extracted OMC and OMS showed that the extracted OM is representative for the OM in the sediment but due to the polar nature of extractant (water) the OM is enriched in the more easily extractable OM, including carbohydrates and protein-derivatives.

Additionally, the clayey bulk sediment and extracted OMC were also analyzed by Pyrolysis GC-MS (for the OMS samples the C content in bulk sediments and the amount of extracted OM were too low). In total, 76 and 59 pyrolytic organic compounds were identified in bulk sediment and extracted OMC, respectively. These compounds were grouped into 13 classes (e.g., carbohydrates, N compounds, (alkyl)benzenes, *n*-alkanes, lignin, etc.) (Table S2.4) (Ninnes et al., 2017; Tolu et al., 2017). In addition to the decrease in the number of identified organic

compounds (from 76 to 59) in the water extract that was also freeze-dried, resuspended and filtered, the Pyrolysis-GC/MS data showed, similarly to the ^{13}C-NMR and FTIR findings, that carbohydrates, N compounds (originating from proteins and degradation products of proteins and chlorophylls) and carboxylic acids got enriched during the extraction, whereas the abundance of more complex molecules such as polyaromatic compounds, (alkyl)benzenes, *n*-alkanes, *n*-alkenes, and lignin decreased. This is probably a result of the differences in extractability of more polar vs. less polar (more hydrophobic) compounds.

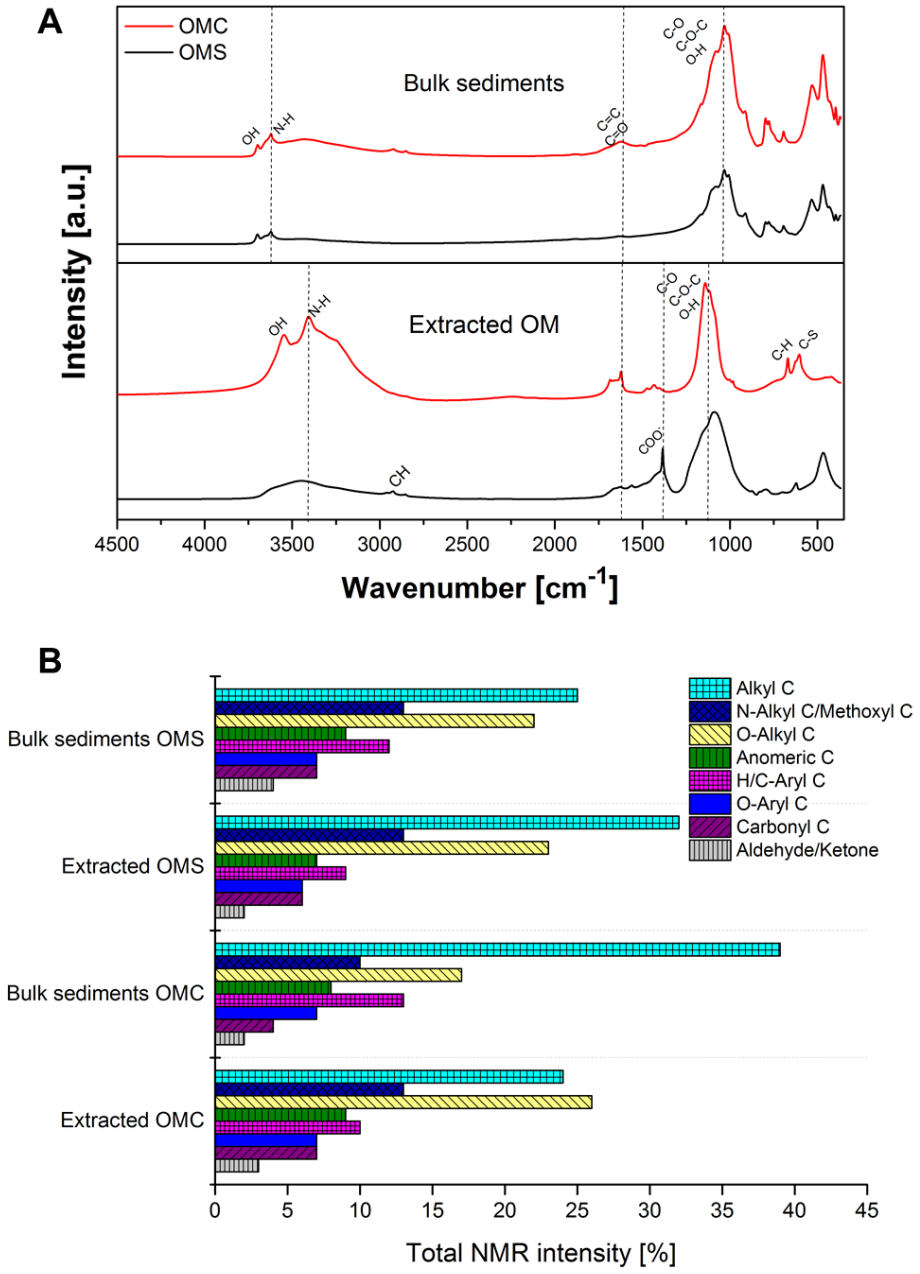

Figure 2.1. Characterization and comparison of the OM extracted from the Van Phuc aquitard (clayey silt) and aquifer (sandy) sediments, i.e. OMC and OMS. A) FTIR spectra with assigned peaks and potential C compounds: C-O, C-O-C, O-H (polysaccharides), COO- (amino/fatty acids), OH (cellulose), C=C, C=O (lignin-derivatives), and B) shows the distribution of C-containing structural components quantified by ^{13}C-NMR analysis.

However, although the relative abundance of some compounds changed during the extraction, the OM obtained by anoxic water extraction yields OM that is representative for the OM present in the bulk sediments justifying its use in the microcosms as environmentally relevant *in-situ* OM.

Our spectroscopic analyses as well as our visual evaluation of the sediments (Figure S2.1C) showed the presence of some plant residues, suggesting a higher presence of lignin- and cellulose-related compounds in OMC compared to OMS. Previous analysis of clayey silt sediments from the same site identified compounds such as C_{20}–C_{34} *n*-alkanes, C_{14}–C_{34} *n*-alkanoic acids, C_{20}–C_{31} ω- hydroxy alkanoic acids and C_{16}–C_{31} *n*-alkanols (Al Lawati et al., 2012), also indicating the presence of plant-derived OM (Xing et al., 2011). Overall this implies that more lignin and cellulose related compounds were present in OMC than in OMS. In combination with our visual observation of the material (where remaining plant-derived organic structures were observed) this suggests that OMC is more immature, plant-derived OM compared to OMS. Overall, on the one hand the abundance of OM is higher in the upper clayey silt, but the bioavailability of this C seems to be lower due to the presence of more complex molecules and not fully degraded plant material. On the other hand, the sandy sediments are characterized by a very low organic C content. However, this C potentially has a higher bioavailability resulting from its more advanced degradation stage and the presence of amino acids and carboxylic acids which points towards a microbial signature (Berggren et al., 2010; Rowland et al., 2007).

2.4.2 Effect of different C sources on Fe(III) mineral reduction and As mobilization

To determine the effect of different C sources on As mobilization we set up microcosms with oxidized As-bearing sediments. We were particularly interested in the effect of the OM from the overlaying clayey silt sediments (OMC) that was suggested to be transported downwards into the OM-poor sandy sediments to drive Fe(III) reduction and As mobilization in these layers (Lawson et al., 2013). The Fe and As contents in these sediments used for the microcosm incubations were determined by XRF to be 1.6 mg/g and 5.5 µg/g, respectively, while the TOC was rather low (0.15±0.002 wt%). Mineralogical analysis with X-ray diffraction (XRD) revealed goethite, hematite and siderite as the main Fe minerals and to a smaller extent magnetite and greigite (M. Schneider, unpublished data).

All our microbially-active microcosms showed Fe(III) reduction while biologically inactive microcosms (treated with sodium azide) that were supplied with acetate/lactate (CON-) showed no significant changes in dissolved Fe, Fe(II) in sediments, and dissolved As over 100 days of

incubation demonstrating that OM was fueling microbially mediated Fe(III) reduction (Figure 2.2). However, the extent and rates of Fe(III) reduction and As mobilization differed between various C sources supplied.

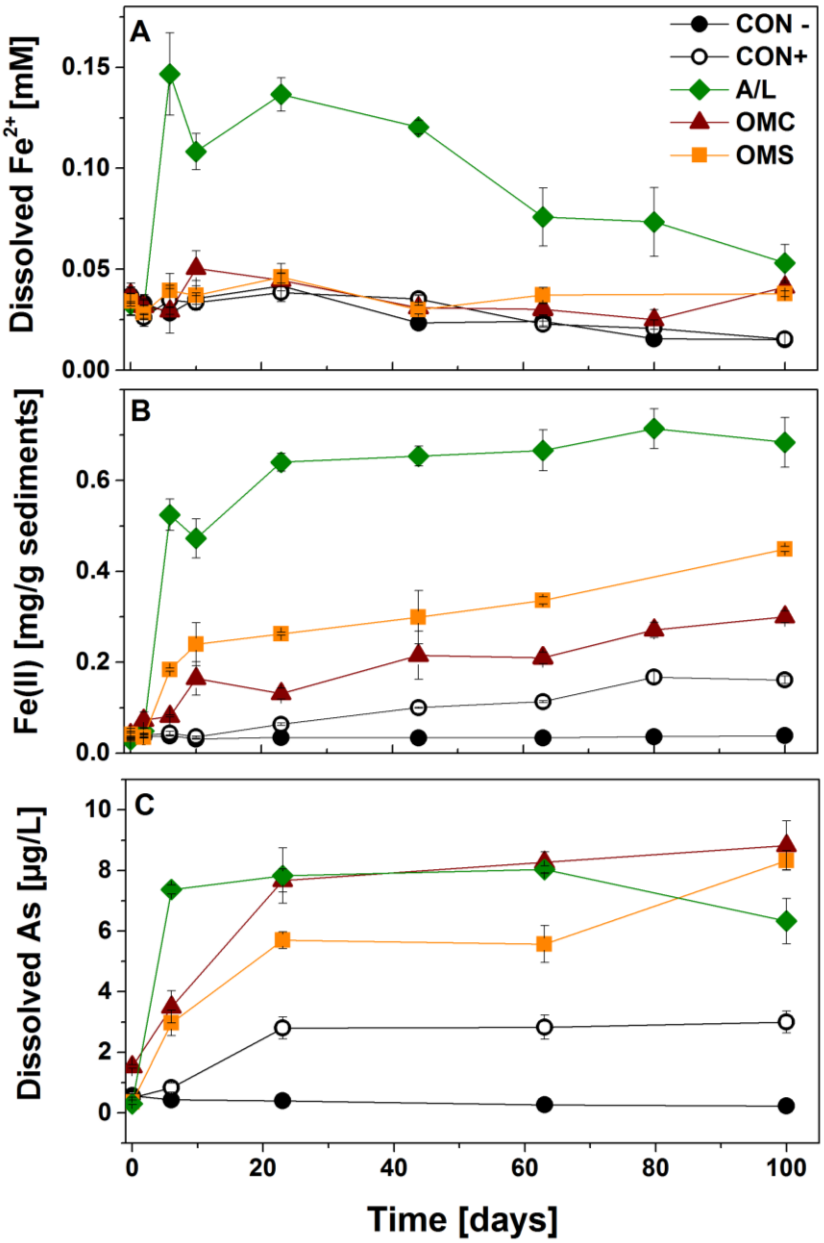

Figure 2.2 Changes of Fe(II) in the sediment, dissolved Fe^{2+} and dissolved As over 100 days of incubation of As-bearing sediments in microcosms supplied with different C sources. A) concentration of Fe(II) in the sediment quantified by 1 h digestion with 6 M HCl, B) concentration of aqueous Fe^{2+}, C) dissolved As (please note that this is the As mobilized from 1 g of sediments into 5 mL volume of artificial groundwater). Biotically active control without additional C (CON+), abiotic control supplied with 160 mM NaN_3 in order to inhibit microbial activity and amended with acetate/lactate (CON -), and three microbially active setups amended with different C sources: OM extracted from clayey silt sediments (OMC), OM extracted from sandy sediments (OMS), acetate/lactate (A/L), at 12 mg C/L each. Error bars represent standard deviation from 3 vials. Each vial was measured in triplicate.

The highest concentration of Fe(II) in the sediments was recorded in A-/L-amended microcosms (Figure 2.2A) where it reached 0.52 mg/g sediment after 6 days and 0.64 mg/g sediment after 23 days, remaining at this level until the end of the experiment, when we detected almost 0.7 mg Fe(II)/g (43±3.5% of the total Fe in the sediment; the values of % reduction were calculated using the Fe(II) extracted from the sediment divided by the sediment Fe content determined by XRF).

When microcosms were supplied with *in-situ* OM, less Fe(II) was formed (ca. one third to half of the Fe(II) formed in the A-/L-amended setups). However, Fe(II) was steadily produced during the experiment until the end of incubation (100 days). The Fe(II) remained completely in the solid phase, reaching 0.08 and 0.18 mg Fe(II) per g sediment after 6 days and 0.3 and 0.45 mg/g after 100 days in OMC and OMS setups, respectively, corresponding to 19±0.8% and 28±0.3% of the total Fe present in the sediment. These results showed that statistically more Fe(III) (*t*-test, $p<0.005$) was reduced (28±0.3%) by OMS compared to microcosms supplied with OMC (19±0.8%). This might be due to the higher bioavailability of OMS (increased content of amino acids and carboxylic acids) compared to OMC, supporting our hypothesis that the identity and composition of the OM are the factors deciding about its potential as C-source for Fe(III)-reducing microorganisms. It has to be noted that accumulation of Fe(II) in the sediments also occurred in the non-C-amended biotic control (CON+) sediments, although to a lower extent (0.16 mg/g; corresponding to 9% of the total Fe), suggesting that the indigenous microbial community used some of the carbon that was available within the sediments. Generally, in the CON+ microcosms, where some of the *in-situ* OM was mobilized and obviously also was bioavailable, similar trends for Fe(III) reduction were observed as in OMC and OMS setups. Similar Fe(III) reduction patterns could indicate that the extracted OM is qualitatively closer and more representative to sedimentary NOM than acetate and lactate. However, ultimately in the end of the experiment significantly less Fe(II) was produced in CON+ compared to OMC- (*t*-test, $p<0.005$) and OMS-amended microcosms (*t*-test, $p<0.005$) due to the lower abundance of the native sedimentary C that was present in the CON+.

In microbially-active acetate-/lactate-amended microcosms, Fe(II) was produced and released as dissolved Fe^{2+} into solution, reaching its maximum after 6 days (0.15 mM; i.e. 2.5% of the total Fe in the sediment) followed by a steady decrease until the end of the experiment to 0.05 mM (Figure 2.2B). In microcosms supplied with OMC and OMS Fe^{2+} was not released into solution. Our data showed that in microcosms supplied with OMC and OMS, dissolved Fe^{2+} stayed at a similar level as in the biotic and abiotic controls (CON+ and CON-) suggesting that the formed Fe(II) remained as either sorbed Fe(II) or Fe(II) mineral in the sediments. The

saturation index for different minerals was calculated using PhreeqC in order to explain the lack of Fe^{2+} release in microcosms supplied with *in-situ* OM (see Table S2.5). The calculation showed that no siderite precipitation is expected. Therefore, the lack of Fe^{2+} mobilization could be due to adsorption of Fe(II) on the remaining poorly crystalline Fe(III/II) minerals (Kocar et al., 2006) or formation of NOM-Fe complexes could have prevented Fe^{2+} mobilization. It was previously shown that some functional groups such as carboxyl groups, which were also present in the extracted OM used in our study, are particularly prone to create complexes with Fe(II) at neutral pH (Daugherty et al., 2017).

Quantification of dissolved As showed that trends in As mobilization did not fully correlate with Fe(III) reduction in the sediments (Figure 2.2C). By the first 6 days of incubation dissolved As was found to be higher in A-/L-amended setups than in OMS and OMC setups, where almost 8 µg/L dissolved As was released from 1 g of sediment, i.e. a mobilization of 0.7% of the total As present, compared to less than 4 µg/L As in OMS and OMC setups (the %-values of mobilized As were calculated using dissolved As concentrations in the 5-ml-volume at given time points divided by the sedimentary As content determined by XRF). The concentration of dissolved As decreased after 60 days in A-/L-setups (to 6.3 µg/L at the end of incubation), which might be related to the decrease of aqueous Fe^{2+}, possible formation of secondary Fe minerals (that are not considered in our saturation index calculation, Table S2.5) and As co-precipitation (Muehe et al., 2013). A similar rapid Fe(III) reduction and As mobilization followed by As immobilization due to co-precipitation with secondary minerals has been shown for West Bengal sediments amended with acetate (Héry, M. et al., 2010) and glucose-/lactate-amended As-contaminated soils (Chatain et al., 2005). Despite lower extents of Fe(III) reduction, ultimately (day 100) a higher As concentration was recorded in the presence of OMC (8.3±0.3 µg As/L; *t*-test, $p<0.005$) and OMS (8.8±0.8 µg As/L; *t*-test, $p<0.005$) compared to A/L setups, corresponding to mobilization of 0.75 and 0.8% of the total As, respectively. On the one hand, this higher As concentration despite lower Fe(III) reduction could be due to competitive sorption of the OM and As. It is known that organic compounds such as citrate or humic acids can decrease adsorption of phosphate to soil and to Fe(III) minerals such as goethite (Fontes et al., 1992; Geelhoed et al., 1998). As(V) can be considered as an analog of phosphate (Yong et al., 2003), and therefore OM could affect As(V) sorption, but also As(III) sorption, through competition for reactive surface sites and could lead to desorption of As. On the other hand, OM can change As speciation through redox reactions (Redman et al., 2002; Wang & Mulligan, 2006) and formation of binary and ternary complexes with Fe and As (Sharma et al., 2010). Such dissolved NOM-As-Fe complexes can increase the mobility of As, resulting in

increased aqueous As concentrations in groundwater (Breault et al., 1996; Redman et al., 2002). Overall, our study demonstrated that *in-situ* OM (including OM from the aquitard that can potentially be mobilized) can trigger microbial Fe(III) reduction and can contribute to As release. Although initially (until 60 days of incubation) more As was present in solution in microcosms supplied with OMC compared to OMS, the final As concentration (8 µg As/L) was the same for microcosms amended with both types of OM. It has to be noted that although 8 µg As/L might seem to be insignificant, the water to sediment ratio in our microcosms (5:1 wt/wt) was much higher compared to the one in the aquifer (1:8 (wt/wt) assuming a porosity of 25% and a sediment density comparable to quartz) (Farhana S. Islam et al., 2004). Under these conditions the concentration of 8 µg As/L from our experiment would be equivalent to a concentration of 352 µg As/L in the field. This is similar to the concentration measured in contaminated Holocene groundwater at our field site in Van Phuc, suggesting that an important fraction of the mobilized As could be mobilized as a consequence of microbial oxidation of *in-situ* OM coupled to reduction of As bearing Fe(III) minerals.

This observation potentially shows that this type of C can more efficiently release As sorbed to Fe(III) minerals but at the same time be less available for Fe(III)-reducing bacteria.

2.4.3 Microbial key players and activities in Fe(III) reduction and As mobilization

Microbial community analyses were used to unravel the influence of the investigated carbon sources on the microbial community structure and to identify potential microbial key players involved in Fe(III) reduction and As mobilization. Based on qPCR, A/L initially supported vigorous growth of bacteria, reaching $>3.0x10^6 \pm 3.5x10^5$ bacterial 16S rRNA gene copy numbers per g sediment within the first 10 days of the incubation (Figure 2.3A). However, A/L was quickly consumed leading to a decrease (ca. 90%) of the bacterial abundance to $2.4x10^5 \pm 5.0x10^4$ 16S rRNA gene copies per g sediment at the end of the incubation. In contrast, when microcosms were supplied with the *in-situ* OM, the abundance of the bacterial population remained stable in the OMS incubation with $1.5x10^6 \pm 8.6x10^4$ bacterial 16S rRNA gene copy numbers per g sediment and doubled from $1.5x10^6 \pm 1.9x10^5$ to $3.4x10^6 \pm 4.7x10^5$ bacterial 16S rRNA gene copy numbers per g sediment after 100 days in the OMC incubations. Also in the non-C-amended biotic control setups (CON+) an increase of bacterial 16S rRNA gene copy numbers per g sediment was observed over time (from $4.2x10^5 \pm 3.1x10^4$ to $1.8x10^6 \pm 1.6x10^5$ after 100 days), confirming our observations of slower degradation of intrinsic NOM in sediments and therefore slower Fe(III) reduction. On the contrary, archaea seemed to be less selective for the C type. The 16S rRNA gene copy numbers of archaea ranged between $2.4x10^4$

and $3.4x10^4$ per g sediment after 10 days in all treatments (Figure 2.3B). Over time the archaeal population increased in all setups, most notably in the OMC-amended microcosms where 16S rRNA gene copy numbers per g sediment increased by more than one order of magnitude, i.e. from $2.4x10^4 \pm 1.1x10^4$ to $3.5x10^5 \pm 2.0x10^4$, after 100 days.

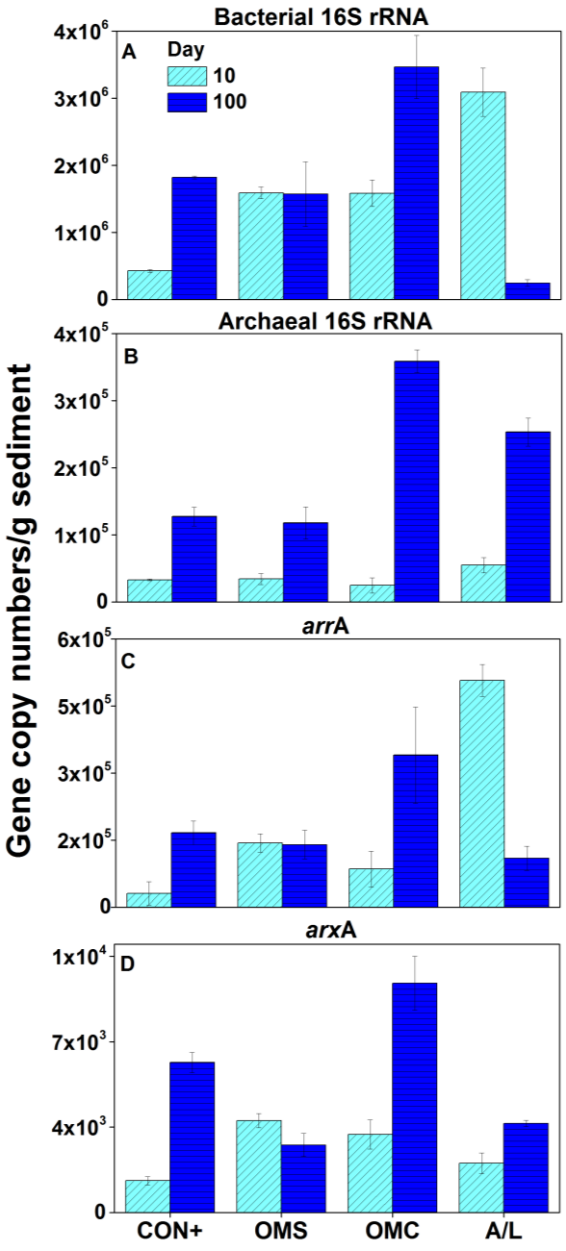

Figure 2.3 Quantitative PCR analysis of A) bacterial 16S rRNA gene, B) archaeal 16S rRNA gene, C) arsenate reductase gene (*arr*A) and D) anaerobic arsenite oxidase (*arx*A) gene copy numbers after 10 and 100 days of incubations with various C sources. Biotically active control without additional C (CON+), and three microbially active setups amended with different C source: OM extracted from clayey silt sediments (OMC), OM extracted from sandy sediments (OMS), acetate/lactate (A/L), at 12 mg C/L each. Error bars represent standard deviation from 3 measurements.

Changes in the microbial population based on 16S rRNA gene copies, particularly bacteria, could indicate that less bioavailable C (and thus more persistent to degradation) such as NOM is consumed much slower. This carbon source could, therefore, last longer compared to simple fatty acids, supporting a higher abundance and a higher diversity of microorganisms on longer time scales. Due to slower consumption of NOM, the Fe(III) reduction was also slow, although, continuously increasing over the whole incubation period and contributing to As mobilization. To investigate the presence of microorganisms with the potential ability for As(V) reduction and As(III) oxidation, we subsequently used qPCR to quantify arsenate reductase genes (*arr*A) and anaerobic arsenite oxidase genes (*arx*A) (Figure 2.3C and 2.3D) that were previously detected in As contaminated environments (Silver & Phung, 2005; Zargar et al., 2012). The *arr*A gene was detected in all microcosms, although at one order of magnitude lower than bacterial 16S rRNA gene copy numbers (Figure 2.3C). After 10 days of incubation, the bacterial 16S rRNA/*arr*A gene ratio was highest in OMC (18:1), followed by OMS (11:1) and lowest in A-/L-setups (6:1), suggesting that microorganisms with the potential ability for As(V) reduction were particularly present in the A-/L-setups. The *arx*A gene copy numbers were two orders of magnitude lower compared to *arr*A genes and 3 orders lower compared to bacterial 16S rRNA genes (Figure 2.3D). Generally, for all treatments except OMS, the number of *arr*A and *arx*A gene copies increased over time which might point towards an increasing potential for As(V) reduction and As(III) oxidation. Based on *arx*A and *arr*A gene abundance, microorganisms with the potential ability to affect the redox state and fate of As are present in our microcosms as well as in the aquifer (unpublished data) and their abundance may change depending on the supplied C type.

To further identify potential key microbial players involved in Fe(III) reduction and As cycling, 16S rRNA gene amplicon sequencing was performed from the original sediments and the sediments supplied with different carbon sources after 10 and 100 days of incubation (Figure 2.4). Alpha diversity estimators based on the Shannon, Pielou E, Faith Pd indices indicated that, after 10 and 100 days, generally higher diversity was observed in the CON+, OMC- and OMS-amended sediment compared to A-/L-amended sediment (Table S2.6). *In-situ* OM might therefore favor more diverse taxa rather than single microbial key players that could be more competitive in utilizing simple C compounds (i.e. acetate/lactate). It is worth mentioning that generally in all treatments the microbial diversity decreased compared to the original sediment. As expected, alpha diversity indices of CON+ after 100 days of incubation were comparable to that in OM. This is most likely due to the fact that natural sediments contain C similar to the one we have extracted, that might become more available when sediments are disturbed but in

lower concentration. Therefore, microbial diversity in all treatments with NOM (including CON+) supported growth of similar taxa, whereas, bioavailable acetate/lactate (A/L) favored fewer microbial taxa (mainly *Geobacter*).

In the natural sediment, microorganisms belonging to *Sulfuritalea* (potential sulfur-oxidizers) (Watanabe, T., et al., 2017) were the most abundant group of microorganisms, representing >10% 16S rRNA relative gene sequence abundance. Other abundant taxa were *Moraxellaceae* (5%), potential arsenite-oxidizing *Hydrogenophaga* (4%) (vanden Hoven & Santini, 2004), and potential ammonia-oxidizing archaea affiliating with *Nitrososphaeraceae* (3%) (Pelissari et al., 2017). Within 100 days of incubation, these microorganisms notably decreased their relative 16S rRNA gene sequence abundance or almost completely disappeared in all treatments, possibly due to the lack of substrates necessary for their growth. The most notable enrichment was observed for *Geobacter*, a well-known Fe(III)-reducer (F. S. Islam et al., 2005), with an initial relative 16S rRNA gene sequence abundance of <0.5%, that increased within 10 days 58, 68 and 136 times (to 29%, 34% and 68%) in CON+, OMS and A/L microcosms, respectively. After 100 days, the relative 16S rRNA gene sequence abundance of *Geobacter* dropped to 5% in CON+, remained at ca. 36% in OMS, and still represented 52% of the total microbial community in A-/L-amended microcosms. Clearly, in these setups *Geobacter* was using acetate as an e^- donor and C source most efficiently (acetate was consumed after 10 day), leading to a rapid increase to 68% in its relative abundance after 10 days compared to its initial relative 16S rRNA gene sequence abundance, followed by decrease to 52% after 100 days. In the non-C-amended biotic control (CON+), *Geobacter* related sequences were also abundant, in particular at the beginning of the incubation. Although the relative abundance of *Geobacter* after 10 days of incubation was 30%, no Fe(III) reduction was observed suggesting that the available C was sufficient to sustain viability of these cells to some extent, but did not lead to significant Fe(III) reduction. Besides *Geobacter*, the only other known Fe(III)-reducer *Geothrix* (Nevin & Lovley, 2002) was found at a very low abundance (<0.5%) in all treatments except for CON+ where it represented 1.3% 16S rRNA relative gene sequence abundance after 100 days suggesting its rather marginal role in Fe(III) reduction.

In contrast, in the OMC setups *Geobacter* was enriched in relative 16S rRNA gene sequence abundance only to a lower extent, representing 7% of the microbial community after 10 days and 13% after 100 days. This could indicate that the added OMC was less accessible to this group of microorganisms than acetate, lactate or OMS. Instead, the OMC appeared to be a more suitable carbon source for other microorganisms that increased in relative 16S rRNA gene sequence abundance within 10 days, such as *Erysipelothrix* (10.2%), *Dechloromonas* (9%) and

Prolixibacteraceae (13%), although their abundance decreased by the end of the experiment to 7.6, 2.4 and 0.2%, respectively. In OMS-amended microcosms, *Propionivibrio* and *Desulfotomaculum* were enriched to 14% and 18% relative 16S rRNA gene sequence abundance, respectively. However, their abundance also dropped to 4.6% and 0 at the end of the experiment suggesting they were not involved in Fe(III) reduction directly. In A-/L-amended microcosms, besides *Geobacter* only *Azoarcus* increased its relative 16S rRNA gene sequence abundance from 0.5% at the beginning to up to 12% after 100 days.

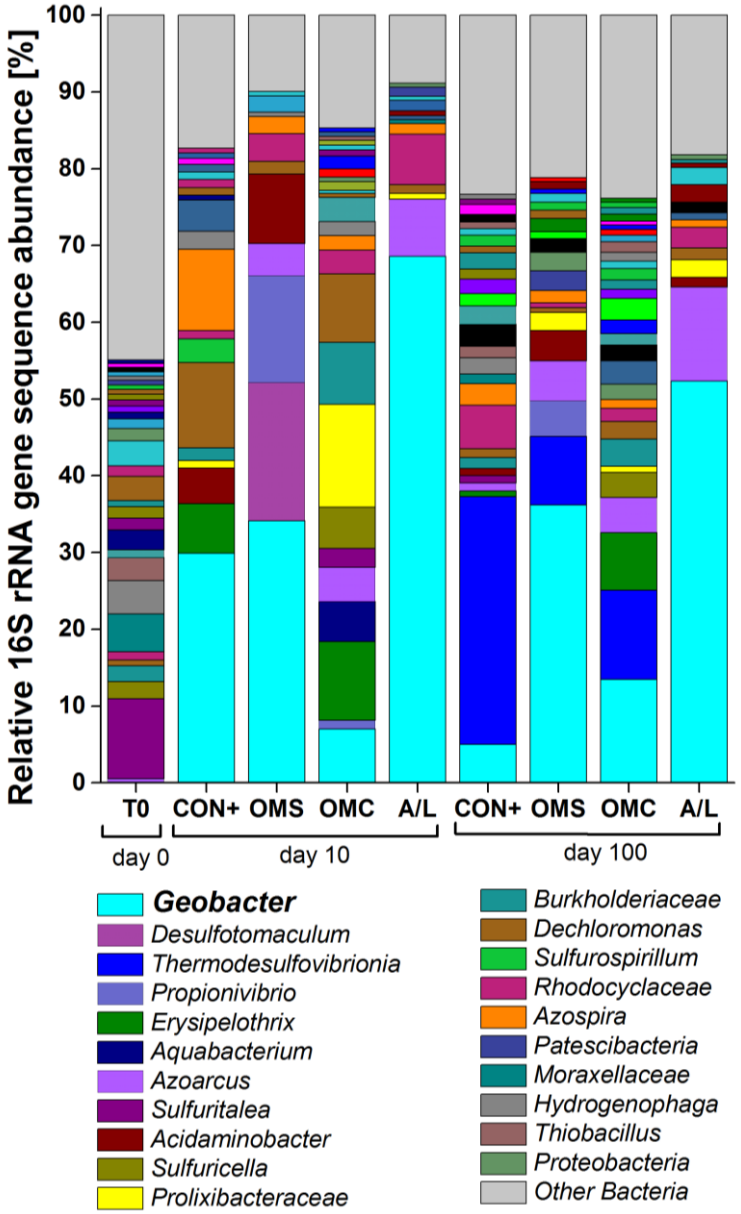

Figure 2.4 Changes in microbial community composition within 10 and 100 days of incubation with various C sources. The presented taxa were analyzed at genus level (and labelled with highest descriptive taxonomic level) and minimum abundance level of 0.5%. Biotically active control without additional C (CON+), abiotic control supplied with 160 mM sodium azide in order to inhibit microbial activity and amended with acetate/lactate (CON-), and three microbially active setups amended with different C source: OM extracted from clayey silt sediments (OMC), OM extracted from sandy sediments (OMS), acetate/lactate (A/L), at 12 mg C/L each. T0 represents the initial microbial community at the beginning of the experiment.

While most of taxa decreased their relative 16S rRNA gene sequence abundance, *Azoarcus*, as one of very few taxa increased its abundance in all treatments, pointing towards its involvement in C utilization and Fe(III) reduction. Also, *Thermodesulfovibrionia*, microorganisms known for reduction of sulfate and other sulfur compounds (Matsuura et al., 2016), appeared abundant in the end of the incubations reaching up to 32% in CON+, 11% in OMC and 9% in OMS; however, this taxon was not detectable in A-/L-amended microcosms. *Geobacter*-related microorganisms were previously found in Van Phuc sediments (Al Lawati et al., 2012) as well as in other As-contaminated aquifers where Fe(III) reduction is a significant terminal electron-accepting process (Fontes et al., 1992; F. S. Islam et al., 2005; Kim et al., 2012; Lear et al., 2007), however, its *in-situ* abundance was rather low. In our experiment, the oxidation of bioavailable acetate supported growth of this microorganism fueling microbial Fe(III) reduction. Consequently, fast Fe(III) reduction rates occurred during the first few days of incubation as well as a significant increase in bacterial 16S rRNA gene copy numbers (Figure 2.3A). However, once acetate was depleted, Fe(III) reduction stopped and the number of bacterial 16S rRNA gene copy numbers (including *Geobacter*) decreased to only 8% of the initial value at day 10. Although *Geobacter* also enriched in the presence of natural OM, other taxonomic groups such as *Prolixibacteraceae*, *Erysipelothrix*, *Dechloromonas*, *Propionivibrio*, *Desulfotomaculum*, *Azoarcus* and *Thermodesulfovibrionia* enriched as well. Some of these taxa were previously reported to be present in As-contaminated environments, suggesting their potential direct or indirect role in As cycling (Nevin & Lovley, 2002). Therefore, our results demonstrate that using bioavailable C such as acetate/lactate favors growth of specific microorganisms (i.e. *Geobacter*). However, based on VFA analysis of the porewater, we know that acetate and lactate can be found in the aquifer only sporadically and at concentrations below a few μM, therefore these VFA are probably not the main carbon sources *in-situ*. In contrast, *in-situ* OM enriched diverse taxa and maintained the microbial population for much longer (whereas in the A-/L-amended setups, after 10 days when acetate was already consumed, the cell numbers decreased drastically), suggesting that an increasing complexity of OM might stimulate more diverse microbial communities for much longer in the groundwater aquifer, contributing to slower but prolonged Fe(III) reduction and As mobilization.

2.5 Environmental Implications

Our study demonstrates that the identity and reactivity of the organic matter controls the rates and extent of Fe(III) reduction and subsequent As mobilization from aquifer sediments under anoxic conditions. Although the commonly used easily bioavailable C-sources such as acetate,

lactate, glucose or lactose are useful as a proxy in simple laboratory experiments, they do not fully represent environmentally relevant OM, particularly when used at very high concentrations. In order to gain a full understanding of the prevalent processes and the microbial community involved in the environment, it is necessary to compare the results with those from *in-situ* OM.

Due to the lower bioavailability of *in-situ* OM, Fe and As biogeochemical transformation processes will be most likely much slower than previously assumed based on the experiments with highly bioavailable C which introduce a bias in estimation of As mobilization. In our study we employed novel approaches of using C that is qualitatively more representative of *in-situ* OM and help to better estimate Fe(III) reduction and As mobilization. We showed that OM extracted from the aquifer sediments may serve as a substrate for diverse microbial taxa and sustain their metabolism for much longer while simple C sources such as acetate and lactate may be consumed very quickly leading to decreased abundance and microbial diversity favoring the most competitive microorganisms such as *Geobacter*. However, the *in-situ* OM does not only serve as electron donor for bio-induced Fe mineral transformation but can potentially also be involved in abiotic reactions due to its sorption properties and its capacity to form metal complexes. To better understand the biogeochemical reactions involving NOM, Fe, and As, synchrotron based analysis (XANES) could be used to follow As speciation. Overall, our findings improve the understanding of the fate and cycling of As in groundwater aquifers and provide suggestions for future experiments testing the effect of *in-situ* OM on As mobility.

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2.7 Supporting Information

Materials and Methods

Organic Matter Extraction and Characterization. For total organic carbon (TOC) quantification, sediments were dried at 60°C until weight stability was reached and ground to a fine powder using a mortar and pestle. TOC of dried samples was quantified using a VarioEL C/N analyzer (Elementar).

Excitation-emission matrix (EEM) fluorescence spectra were obtained for 50 mg C/L solutions (dissolved in anoxic MilliQ water) of all extracts using a Fluorometer FluoroMax 4 (Horiba) in a 1 cm quartz cuvette. The excitation wavelength was incrementally increased from 200 to 600 nm while the emission wavelength was recorded from 350 to 700 nm at 1 nm steps.

Fourier-transform infrared (FTIR) spectra were collected using a VERTEX 80v vacuum FT-IR spectrometer (Bruker Optik GmbH). Two mg extracted and freeze-dried OM or dried and finely ground bulk sediment were mixed and homogenized with 248 mg of KBr and hydraulically pressed into small pellet. The FT-IR spectrometer was set up to scan from 400 to 4000 cm^{-1} averaging 32 scans at 1 cm^{-1}.

Solid-state ^{13}C NMR spectra were obtained with a Bruker Avance III HD 400 MHz Wideboard operating at a frequency of 100.63 MHz using zirconium rotors of 4 mm OD with KEL-F-caps. The cross polarization magic angle spinning (CPMAS) technique was applied during magic-angle spinning of the rotor at 14 kHz. A ramped 1H-pulse was used during a contact time in order to circumvent spin modulation of Hartmann-Hahn conditions. A contact time of 1 ms and a 90° 1H-pulse width of 2.2 µs were used for all spectra. The ^{13}C-chemical shifts were calibrated to tetramethylsilane (= 0 ppm) and were calibrated with glycine (176.04 ppm). The relative intensities of the peaks were obtained by integration of the specific chemical shift ranges by an integration routine with MESTRE NOVA. For analysis, the NMR spectra were divided into different chemical shift regions according to Knicker (Kinicker, 2011). The relative C distribution was determined by integrating the signal intensity in different chemical shift regions.

Pyrolysis-gas chromatography/mass spectrometry was performed using from 100 to 500 µg of sediment depending on the samples (and their TOC content) and an oven pyrolyzer equipped with an auto sampler (PY-2020iD and AS-1020E, FrontierLabs, Japan) connected to a GC/MS system (Agilent, 7890A-5975C, Agilent Technologies AB, Sweden). The operating conditions were as described in Tolu et al. (Tolu et al., 2015) (e.g., Py temperature of 450°C) except that a

split ratio of 6:1 was used. Peak integration was done using a data processing pipeline under the "R" computational environment as described in Tolu et al. (Tolu et al., 2015). Peak identification was made using the software "NIST MS Search 2" containing the library "NIST/EPA/NIH 2011" and additional spectra from published studies.

16S rRNA (gene) sequence analysis. Sequencing data were analyzed with nf-core/ampliseq v1.0.0 (Straub et al., 2019) that wraps all analysis steps and software and is publicly available at https://github.com/nf-core/ampliseq. Briefly, primers were trimmed and untrimmed sequences were discarded (<4%) with Cutadapt v1.16 (Martin & Cutadapt, 2011). Adapter and primer-free sequences were imported into QIIME2 v2018.06 (Bolyen et al, 2018), quality checked with demux (https://github.com/qiime2/q2-demux), and processed with DADA2 v 1.6.0 (Callahan et al., 2016) to remove PhiX contamination, trim reads (before median quality drops below 35, that is forward 194, reverse 208), correct errors, merge read pairs and remove PCR chimeras and, ultimately, 3,193 amplicon sequencing variants (ASVs) were obtained. Alpha rarefaction curves were produced with the QIIME2 diversity alpha-rarefaction plugin which indicated that the richness of the samples has been fully observed. A Naive Bayes classifier was fitted with 16S rRNA gene sequences extracted from SILVA v132 (Pruesse et al., 2007) QIIME compatible database 99% identity clustered sequences using the PCR primer sequences. ASVs were classified by taxon using the fitted classifier (https://github.com/qiime2/q2-feature-classifier). 19 ASVs with <1% relative abundance per sample classified as chloroplast or mitochondria were removed and the remaining ASVs had their abundances extracted by feature-table (Pruesse et al., 2007). Alpha diversity indices (Observed ASVs, Shannon's diversity index, Pielou's Evenness, Faith's Phylogenetic Diversity) were calculated on a rarefied count table with the minimum sampling depth of all samples (39,027) with the diversity plugin (https://github.com/qiime2/q2-diversity) within QIIME2.

Quantitative PCR. Total cell numbers of bacteria and archaea were estimated by quantitative PCR (qPCR) (Bio-Rad Laboratories GmbH, Munich, Germany) based on the amplification of the 16S rRNA genes. qPCRs were performed using DNA extracts from 1 g of sediment pooled together from triplicates and SybrGreen® Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on an iQ5 real-time PCR detection system (iQ5 optical system software, version 2.0, Bio-Rad). Plasmid vectors (pCR2.1®, Invitrogen, Darmstadt, Germany) containing a cloned 16S rRNA gene fragment from *Thiomonas* sp. and *Halobacterium salinarum* were used as standards for the quantification of total bacteria and archaea, respectively. Clone Red_A06 As SF and clone Red_B11 As SF were used for arsenate reductase (*arr*A) and anaerobic arsenite

oxidase (*arx*A), respectively. Each qPCR assay was repeated three times with triplicate measurements of each sample per run. Data analysis was done using the iQ5 optical system software, version 2.0 (Bio-Rad, 2006). Cell numbers per 1 g sediments were calculated from the qPCR-derived gene copy numbers.

Table S2.1 Major and trace elements (in mg/L) present in the extracted OM that was resuspended in MilliQ (the OM stock solutions). The elements were determined using MP-AES and the values represent the average of 3 measurements ± standard deviation. BDL = below detection limit.

OM stock	Ca	Mg	Cu	Fe	Na	K	As
OMC	0 ±0	30.5 ±0.03	0.02 ±0.00	0.3 ±0.00	13.4 ±0.03	5.26 ±0.005	BDL
OMS	9.08 ±0.07	15.8 ±0.08	0.002 ±0.00	0.02 ±0.005	0.00 ±0.00	27.7 ±0.08	BDL

Table S2.2 Overview about microcosms including concentrations of all amendments.

Setup	OM from clayey aquitard	OM from sandy aquifer	Acetate and lactate (A/L)	NaN_3
CON-	x	x	1 mM C	160 mM
CON+	x	x	x	x
OMC	1 mM C	x	x	x
OMS	x	1 mM C	x	x
A/L	x	x	1 mM C	x

Table S2.3 List of primers, primers sequences and thermal programs used for quantification of bacterial and archaeal 16S rRNA gene, arsenate reductase gene (*arr*A) and anaerobic arsenite oxidase (*arx*A) gene copy numbers.

Specificity	Standard	Primer	Primer sequence (5´→ 3´)	Thermal program	References
16S rRNA gene *Bacteria*	*Thiomonas* sp.	341F	CCT ACG GGA GGC AGC AG	98°C - 2´; (98°C - 5´´; 60°C - 12´´	Muyzer et al., 1993
		534R	ATT ACC GCG GCT GCT GG	95°C - 1´; 60°C - 1´) x 40; 60 – 95°C - 10´´	
16S rRNA gene *Archaea*	*Halobacterium salina*	Ar109F	ACK GCT GAG TAA CAC GT	98°C - 3´; (98°C - 5´´; 52°C - 12´´	Großkopf et al., 1998 Stahl and Amann 1991
		Ar91 R	GTG CTC CCC CGC CAA TTC CT	72°C - 15´) x 40; 98°C - 1´; 52°C - 1´; 52 – 95°C - 10´´	
Arsenate reductase gene (*arr*A)	clone Red_A06 As SF	arrAF	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYTT	95°C - 2´; (95°C - 30´´; 62°C - 40´´) x 40; 95°C - 1´; 62°C - 1´; (62 – 95°C - 10´´) x 67	Song et al., 2009
		arrAR	CCT GTG ATT TCA GGT GCC CAY TYV GGN GT		
Anaerobic arsenite oxidase gene (*arxA*)	clone Red_B11 As SF	arxAF	TAC GAC TAY CGC AAC RCC AAC	95°C - 2´; (95°C - 30´´; 60°C - 40´´) x 40; 95°C - 1´; 60°C - 1´; (60 – 95°C - 10´´) x 71	Nitzsche, 2015
		arxAR	GGT CTT SGG SSW CTT SGT GCG		

Table S2.4 Relative abundance of compound families obtained from Pyrolysis-gas chromatography-mass spectrometry (Pyrolysis-GC/MS). Due to the low C content of OMS samples, analysis was not possible for these samples.

OM class	OMC Bulk	OMC Extracted
Nr of compounds identified	76	59
Carbohydrates	1.4	4.5
N compounds	1	26
Chlorophyll	3	-
Lignin	2	-
Phenol	0.1	6
Alkyl benzenes	23	12
Polyaromatic	8	4
n-alkanes	28	13
n-alkenes	34	9
Alcohols	-	3
Carboxylic acids	-	8
Other aliphatics	0.3	1.0
S compounds	-	12

Table S2.5 Saturation indices calculated for different minerals using the PhreeqC v3 and minteq.v4 database. The calculation was done for given time points based on the available geochemical data.

	OMC	**OMS**	**A/L**
Day	100	100	6
Saturation indices			
Ferrihydrite	-5.17	-5.19	-4.64
Goethite	-2.48	-2.49	-1.94
Hematite	-2.55	-2.58	-1.48
Siderite	-1.44	-1.49	-0.65
Geochemical data [mg/L]			
pH	7.3	7.3	7.3
CO_2 pp [bar]	0.1	0.1	0.1
Alkalinity	550	550	550
Li	0.0031	0.0028	0.0055
B	0.43	0.31	1.04
Na	29.5	17.8	22.8
Mg	50.3	28.1	29.3
Si	14.1	15.8	32.3
P	0	0	0.2
Cl	36	0	30.7
K	8.8	7.7	6.1
Ca	107	86.7	97
Cr	0.0022	0.003	0.0035
Mn	2.01	2.15	2.5
Fe	1.43	1.13	8.18
Zn	0.196	0.197	0.0383
As	8.8	8.3	7.4
Br	0	0	0
Sr	0.353	0.232	0.208
Mo	0.0012	0.0012	0.0008
Pb	0.005	0.005	0.006

Table S2.6 Observed amplicon sequencing variants (ASVs, a qualitative measure of community richness) and alpha diversity indices; Shannon's diversity index (a quantitative measure of community richness), Pielou's index (a measure of community evenness) and Faith's Phylogenetic Diversity (a qualitative measure of community richness that incorporates phylogenetic relationships between the features), in sediments supplied with different C at the beginning of the experiment, after 10 and 100 days of incubation.

Setup	Day	Observed ASVs	Shannon	Pielou E	Faith Pd
T0	0	1331	8.6	0.827	90.0
CON+	10	743	5.9	0.623	64.8
OMS	10	366	4.7	0.546	35.2
OMC	10	513	6.3	0.699	43.9
A/L	10	417	4.4	0.510	40.2
CON+	100	686	6.6	0.696	57.9
OMS	100	600	6.1	0.662	50.7
OMC	100	605	7.0	0.754	45.2
A/L	100	461	5.3	0.594	41.7

Figure S2.1 Pictures of retrieved cores. (A) Sediments used for OM extraction. Left: aquitard clayey silt sediments (OMC) from 11 m depth; right: aquifer sandy sediments (OMS) from 21 m depth. (B) orange sandy sediments from 30 m depth used for the microcosm incubation experiment. (C) Clayey silt organic matter rich aquitard sediments similar to the ones used for extraction of OMC – please note the brownish-dark patches indicating remaining plant residues.

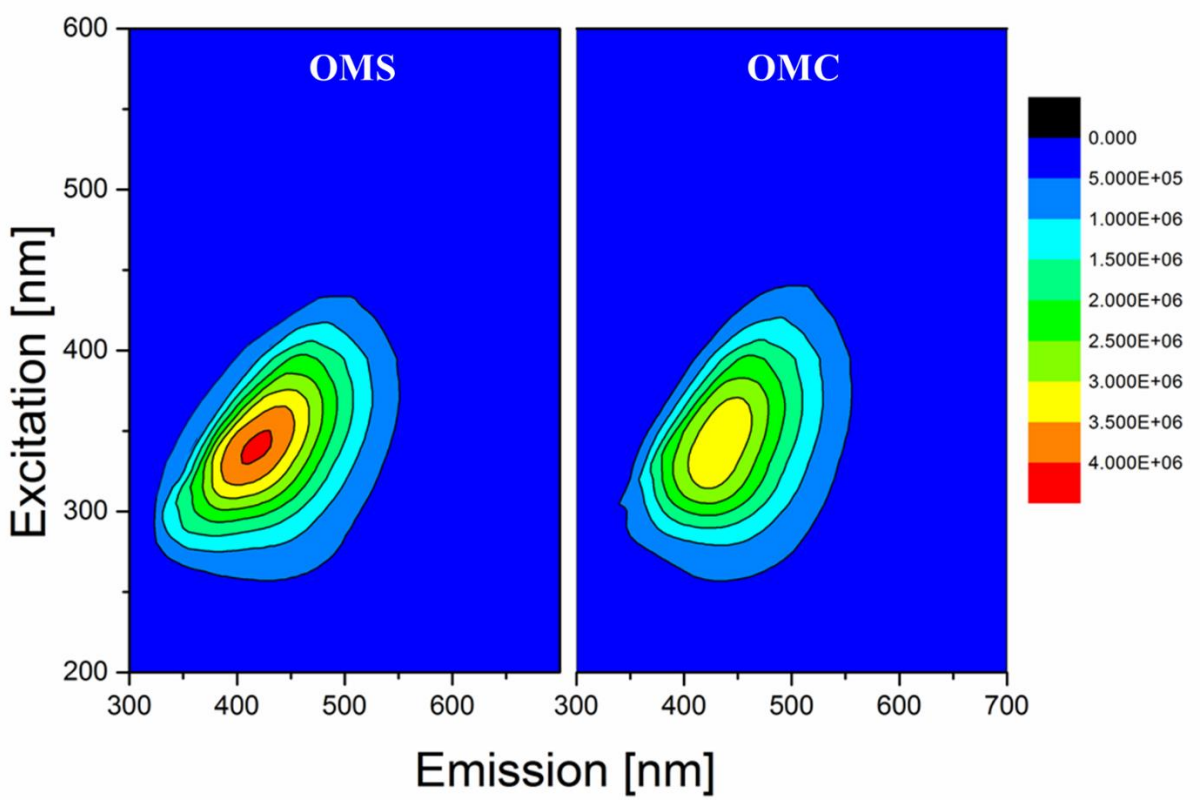
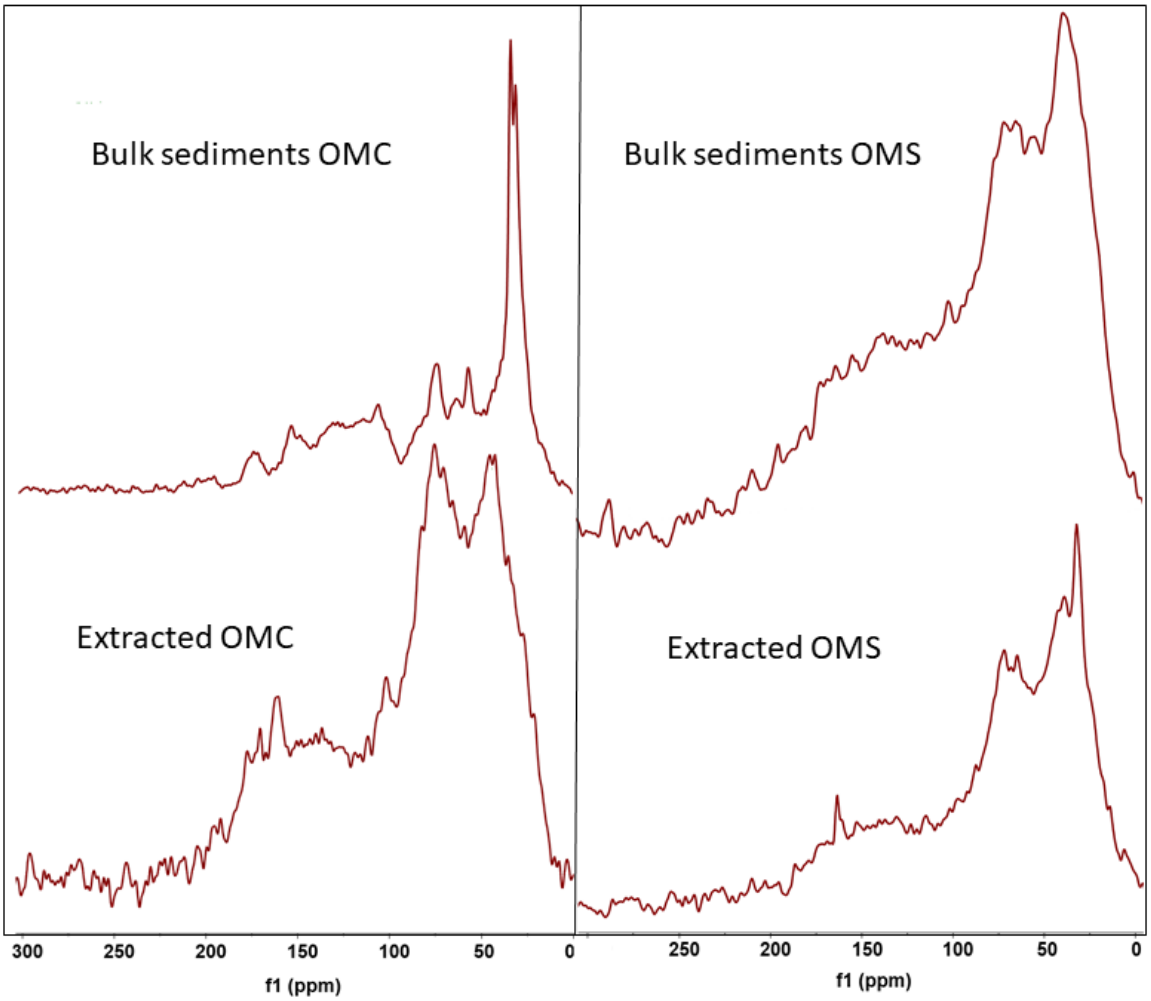


Figure S2.2 Fluorescence spectra (excitation-emission matrices-EEM) of extracted OM from sand (OMS) and extracted OM from clay (OMC). The peaks for both types of OM were located in the same wavelength areas ($\lambda_{Ex/Em}$ = 350/425), suggesting that both C sources have some similar structural components.



ppm	Assignment
0-45	**Alkyl-C**
45-110	**O- and N-alkyl**
45-60	aliphatic C-N, methoxyl
60-90	alkyl-O (carbohydrates, alcohols)
90-110	acetal and ketal carbon (carbohydrates) and some aromatic C
110-160	**Sp^2-hybridized C**
110-140	aryl-H and aryl-C carbons, olefinic-C
140-160	aryl-O and aryl-N carbons
160-220	**Carbonylic-C/carboxylic-C/amide-C**
160-185	carboxyl and amide-*C*
185-220	aldehyde and ketone carbons

Figure S2.3 Comparison of ^{13}C-NMR spectra of bulk sediments OM and extracted OM and tentative chemical shift assignment of various peaks in a ^{13}C NMR spectrum. Modified from Knicker. 2011.

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Chapter 3 – Personal contribution

The sampling campaigned in Vietnam was organized and jointly performed by all AdvectAs project members and coordinated by Dr. Michael Berg. Sediment samples for the microcosm experiment were collected by myself. The hypothesis was formulated by myself and Prof. Dr. Andreas Kappler supported by CH_4 measurements performed by Alex Lightfoot and Rolf Kipfer from Eawag, Switzerland. Experiments were conceptualized, setup and conducted by myself. The data collection was carried out by myself. The ICP-MS analysis was performed by Dr. Emiliano Stopelli. The discussion and analysis of the obtained results were done by myself and Prof. A. Kappler. The manuscript was written by myself with the support of Prof. A. Kappler and revised by all co-authors.

Chapter 3: Arsenic mobilization by anaerobic iron-dependent methane oxidation

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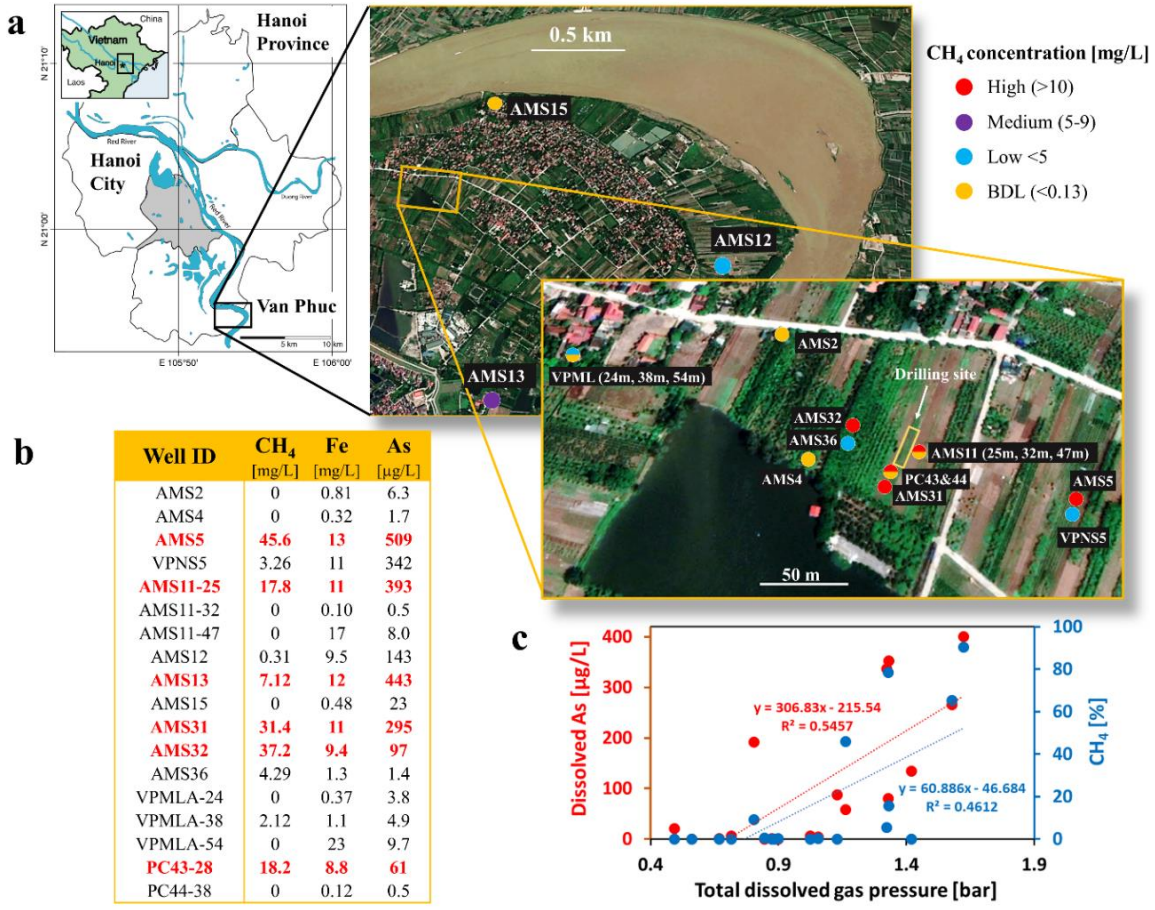
3.1 Abstract

Arsenic groundwater contamination is threatening the health of millions of people worldwide, particularly in river deltas in South and Southeast Asia where geogenic arsenic is released from sediments (Berg et al., 2001, 2007; Charlet & Polya, 2006; Karagas et al., 2015; Smith et al., 2000). In most cases, the release of arsenic (As) was shown to be caused by microbially catalyzed reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals with organic carbon being used as microbial electron and energy source (Glodowska et al., 2020; McArthur et al., 2004; Rowland et al., 2007). Although in many As-contaminated aquifers high concentrations of methane (CH_4) were observed (Buschmann & Berg, 2009; Dowling et al., 2002; Harvey et al., 2002), the role of CH_4 for As mobilization is unknown. Here we demonstrate that CH_4 functions as electron donor for methanotrophic microorganisms and triggers the reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals leading to As mobilization. In microcosms with As-bearing sediments from the Red River Delta amended with environmentally relevant concentrations of CH_4 we found that CH_4 triggers Fe(III) mineral reduction, supports the growth and activity of type-1 aerobic methanotrophs and archaea affiliating with *Candidatus* Methanoperedens, increases the abundance of methane oxidation *mcrA* and *pmoA* genes, and ultimately mobilizes significant amount of As into the water. Therefore, our study provides evidence for a completely new mechanism of As mobilization. In combination with the high concentrations of CH_4 observed in many As-contaminated aquifers this suggests that CH_4-driven As mobilization may occur worldwide contributing to As groundwater contamination.

Exposure to groundwater contaminated with arsenic (As) is a worldwide problem. Arsenical skin lesions are the hallmark of chronic arsenic poisoning appearing within a few years of exposure often leading to skin cancer (Karagas et al., 2015). These symptoms are prevalent in populations living along river floodplains of South and Southeast Asia that rely on shallow groundwater wells for drinking water and irrigation. Arsenic enrichment in shallow groundwater (Berg et al., 2001) has resulted in the so-called ‘worst mass poisoning of human population in history’ (Smith et al., 2000). Generally, Fe(III) (oxyhydr)oxides are common constituents of As-bearing aquifer sediments (Lenoble et al., 2002). Various mechanisms of As release to the groundwater have been suggested including abiotic dissolution and transformation of As-containing Fe minerals including oxidation of arsenic-bearing sulfides and changes in sorption capacity of Fe(III) (oxyhydr)oxide minerals as well as As desorption due to pH variations or by competition with phosphate (Farhana S. Islam et al., 2004). Yet, the most commonly accepted mechanism is microbial reductive dissolution of As-bearing Fe(III)

(oxyhydr)oxides (Harvey et al., 2002; F. S. Islam et al., 2005; McArthur et al., 2004). Fe(III)-reducing bacteria such as *Geobacter*, however, require bioavailable carbon (C) for their activity (Mailloux et al., 2013; Rowland et al., 2007). Previous studies focused on the identity and source of organic compounds used for microbial As mobilization (Al Lawati et al., 2012; Anawar et al., 2006; Lapworth et al., 2008), however, the role of methane (CH_4) remained unexplored. Here we investigated whether CH_4, which is abundant in these aquifers, can also be a potential energy source and drive microbially mediated Fe(III)-mineral reduction leading to As mobilization.

Van Phuc, a village 15 km SE from Hanoi, is known for severe As groundwater contamination (Figure 3.1a), sometimes 60-fold higher than the WHO drinking water limit of 10 µg/L (Eiche et al., 2008). Arsenic-bearing Fe(III) minerals (Berg et al., 2008; van Geen et al., 2013) and groundwater containing high As, Fe and CH_4, low SO_4^{2-}, and no NO_3^- were reported (Figure 3.1b) (Stopelli et al., 2020).



Well ID	CH_4 [mg/L]	Fe [mg/L]	As [µg/L]
AMS2	0	0.81	6.3
AMS4	0	0.32	1.7
AMS5	**45.6**	**13**	**509**
VPNS5	3.26	11	342
AMS11-25	**17.8**	**11**	**393**
AMS11-32	0	0.10	0.5
AMS11-47	0	17	8.0
AMS12	0.31	9.5	143
AMS13	**7.12**	**12**	**443**
AMS15	0	0.48	23
AMS31	**31.4**	**11**	**295**
AMS32	**37.2**	**9.4**	**97**
AMS36	4.29	1.3	1.4
VPMLA-24	0	0.37	3.8
VPMLA-38	2.12	1.1	4.9
VPMLA-54	0	23	9.7
PC43-28	**18.2**	**8.8**	**61**
PC44-38	0	0.12	0.5

Figure 3.1 Sampling sites in Van Phuc village. (a) Distribution of monitoring wells and (b) concentrations of CH_4, Fe and As in groundwater. Wells with high CH_4 are also characterized by high dissolved Fe and As (in red). (c) Total dissolved gas pressure of which the majority accounts for CH_4 correlates with As in groundwater (Lightfoot and Kipfer, unpublished). Groundwater data from Stopelli et al. 2020 (Stopelli et al., 2020).

In-situ gas measurements demonstrated that the total dissolved gas pressure (TDGP) correlates with As concentrations and CH_4 accounts for the majority of detected gases in the groundwater (Figure 3.1c). This makes Van Phuc an ideal location to study Fe(III)-dependent anaerobic methane oxidation and its relevance for As mobilization. Analysis of the *in-situ* microbial community composition by 16S rRNA gene amplicon sequencing in aquifer sediments and groundwater (Figure 3.2) showed that fermenting, methanogenic and methanotrophic microorganisms dominated sediments and groundwater, suggesting that CH_4 cycling occurs in this aquifer.

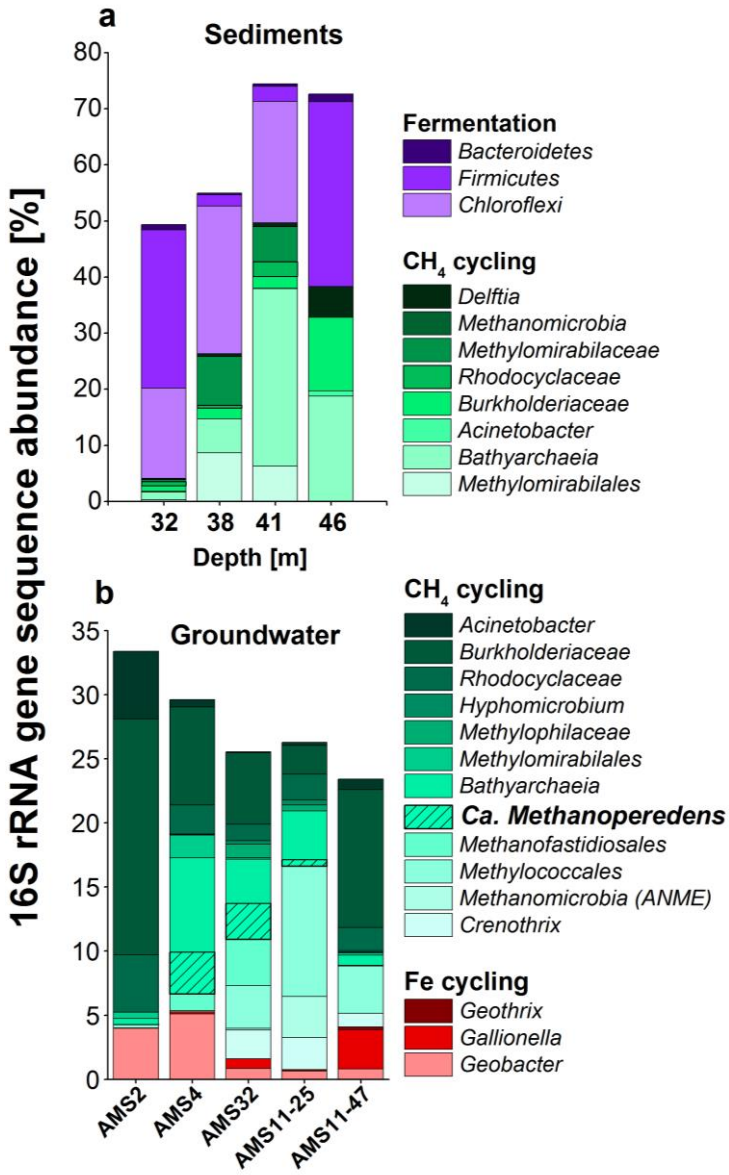


Figure 3.2 Microbial community composition in the Van Phuc aquifer sediments and groundwater. (a) Sedimentary microbial community from different depths across the redox transition zone. The presence of fermenters suggests organic carbon degradation across the aquifer and microorganisms likely involved in CH_4 cycling. (b) Groundwater microbial community from monitoring wells. Relative abundance of microorganisms likely involved in CH_4 cycling compared to microorganisms potentially involved in Fe cycling indicates the importance of CH_4 as electron donor in this aquifer.

Moreover, in some of the groundwater wells we found high abundances (up to 4%) of sequences affiliated to *Candidatus* Methanoperedens, a known Fe(III)-reducing CH_4-oxidizer (Cai et al., 2018; Ettwig et al., 2016).

Using As- and Fe-bearing sediments (Figure S3.1) (~40 m depth), we investigated whether CH_4 can induce microbially driven Fe(III) mineral reduction and As mobilization. Microbially active sediments were amended with artificial groundwater and field-relevant CH_4 concentrations (~45 mg CH_4/L) and compared to abiotic controls (including CH_4) and biotic controls (without CH_4). Iron redox speciation in water and sediments, dissolved and solid-phase arsenic, as well as major ions were monitored over time (SI). Due to the high CH_4 and CO_2 concentrations (simulating *in-situ* field conditions), we were unable to monitor changes in their concentrations (Box S3.1). We characterized the microbial community and identified microorganisms mediating CH_4 oxidation and Fe(III) reduction after 125 days and 220 days of incubation. The results showed that CH_4 triggers Fe(III)-mineral reduction, induces changes in the microbial community composition, increases the abundance of methane-cycle *mcrA* and *pmoA* genes, and ultimately releases As into the water.

Specifically, we found that in the biotic microcosms with CH_4, up to 0.5 mg Fe/g (max. 6% of the total sedimentary Fe) was reduced to Fe(II) until day 120 (Figure 3.3a; S3.2), but no Fe^{2+} was released into the water (Figure 3.3b). Despite the low extent of Fe(III) reduction, As mobilization was observed, reaching nearly 1.5 µg/L (Figure 3.3c), ca. 2% of total sedimentary As. This initial release of As suggests that this As was bound to the easily bioavailable fraction of Fe(III) minerals.

Bacterial 16S rRNA gene copy numbers increased by 4 orders of magnitude during these first 125 days of incubation with CH_4 (Figure 3.4a). While in the original sediment, 16S rRNA gene copy numbers for bacteria and archaea were similar, after 125 days, bacteria dominated over archaea by up to 3 orders of magnitude and *pmoA* genes were abundant (particulate methane monooxygenase, used to target aerobic methanotrophs (Luesken et al., 2011) as well as nitrite-dependent CH_4 oxidation (Welte et al., 2016)). This suggests that, since our experiments were anoxic and did not contain other electron-acceptors (such as NO_x), microorganisms possessing this gene can reduce Fe(III).

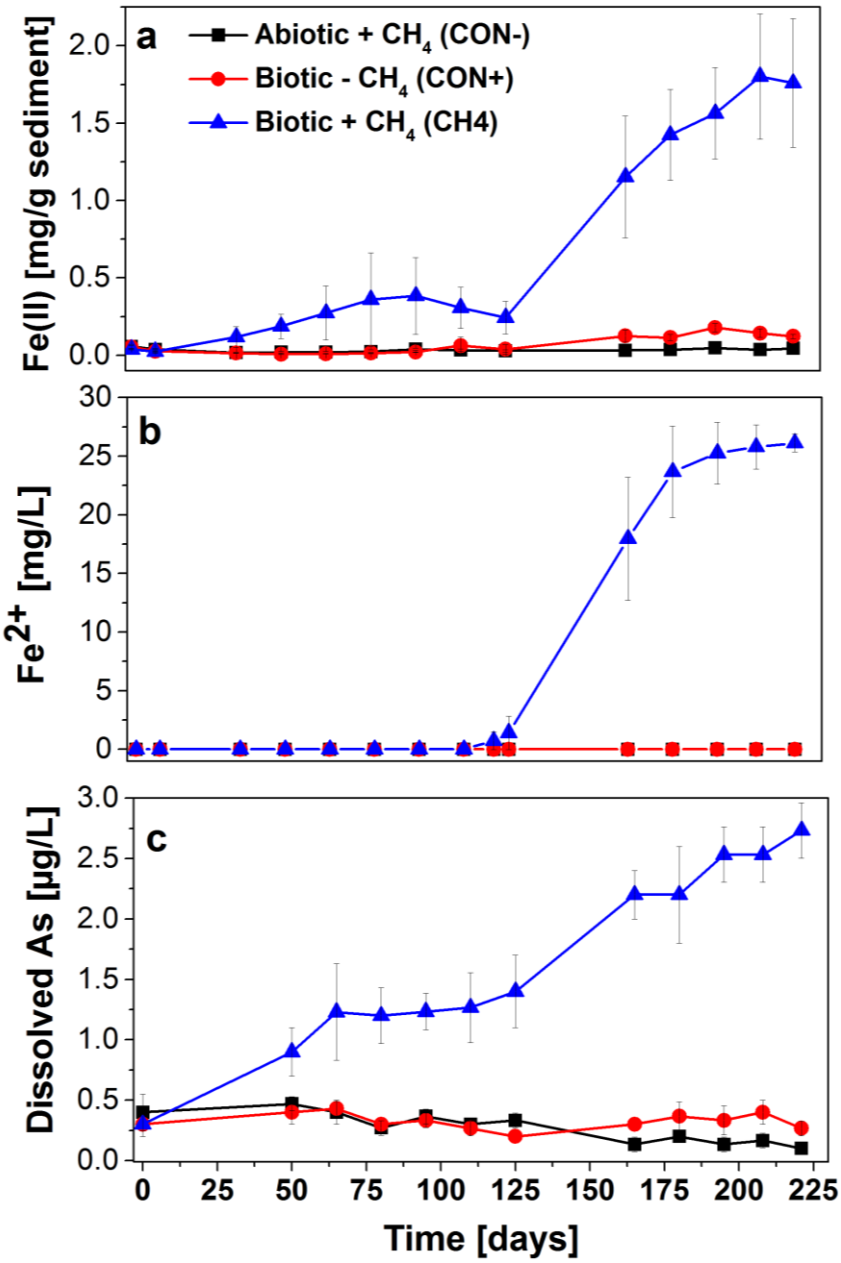


Figure 3.3 Changes in Fe(II) and dissolved As in microcosms. (a) sedimentary Fe(II) (b), dissolved Fe^{2+}, and (c) dissolved As over 220 days of incubation of As-bearing sediments supplied with CH_4 compared to biotic and abiotic setups. Error bars represent standard deviation from 6 microcosms until 125 days and 3 microcosms from days 125-220. Each microcosm was measured in triplicate.

Furthermore, the *mcrA* gene, a marker gene commonly used for the quantification of anaerobic CH_4-oxidizing archaea (Friedrich, 2005) was present in all CH_4-amended microcosms (Figure 3.4a). While the original sediments were dominated by archaea belonging to the phylum *Thaumarchaeota* (22%) and *Bathyarchaeia* (17%) (Figure 3.4b), during the incubation of microcosms supplied with CH_4, microorganisms nearly identical (99%) to *Methylogaea oryzae* jcm 16910 were highly enriched (30-75% relative abundance).

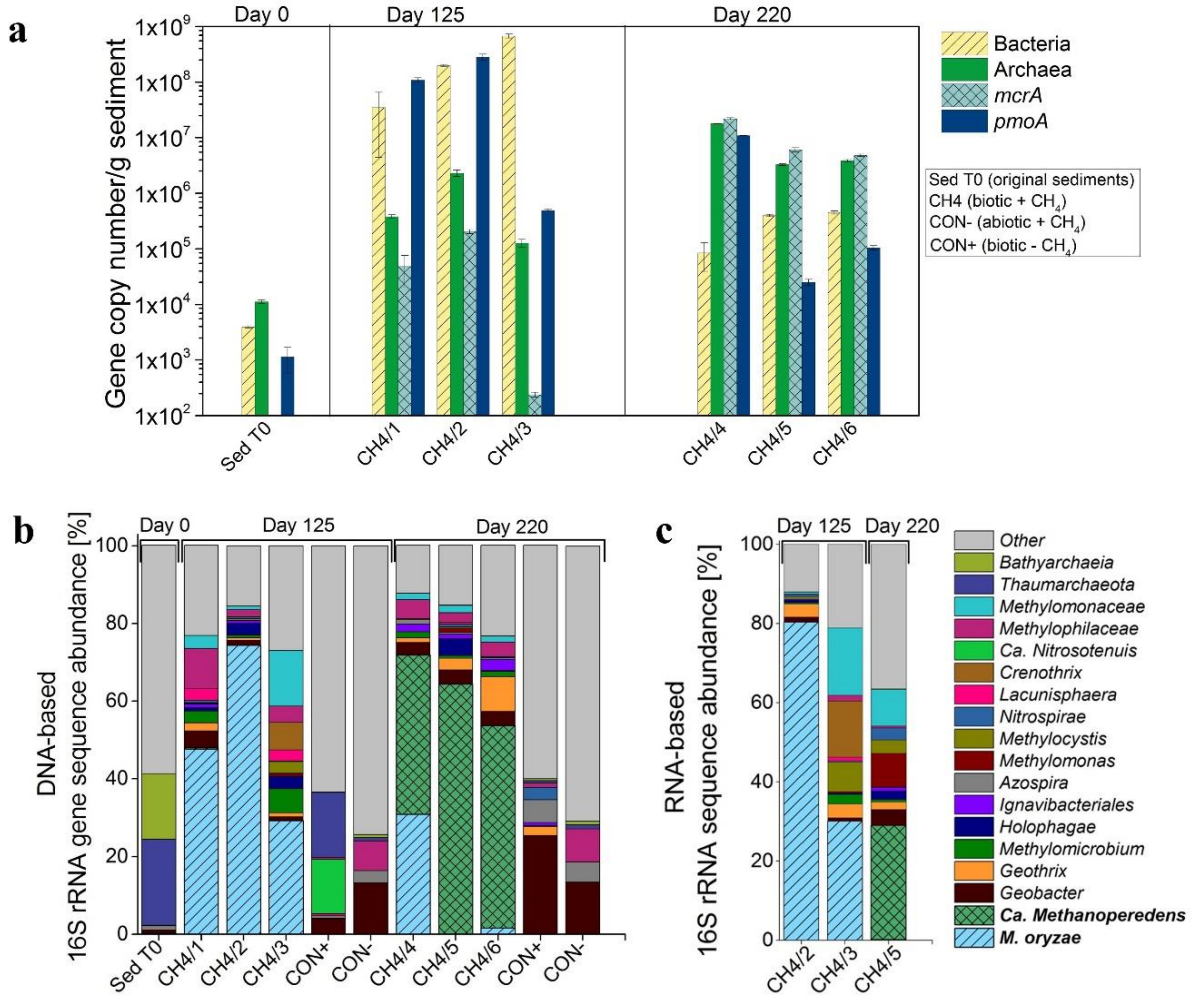


Figure 3.4. Changes in the microbial community composition and gene abundance in microcosms over 220 days of incubation with CH_4. (a) Quantitative PCR of bacterial and archaeal 16S rRNA genes, as well as *mcrA* and *pmoA* genes before the incubation (Sed T0) and after 125 and 220 days of six parallel incubations with CH_4. (b) Abundance based on DNA-based 16S rRNA gene sequencing. (c) Active taxa based on RNA-based 16S rRNA sequencing. The presented taxa were analyzed at genus level (and labeled with highest descriptive taxonomic level) and minimum abundance level of 0.5%.

In the original sediments and in the biotic and abiotic controls they showed very low abundance (<0.05%). RNA-based analysis (Figure 3.4c) showed that *M. oryzae* was also dominating the active microbial community (30-80%), suggesting its capacity to use CH_4 as electron-donor and probably Fe(III) as electron-acceptor. Although *M. oryzae* was characterized previously as aerobic methane-oxidizer (Geymonat et al., 2011), in our experiment it oxidized methane under Fe(III)-reducing conditions. In its genome (IMG ID: 2675903437), we found over 30 protein-coding genes involved in cytochrome c synthesis and 19 protein-coding genes for Fe-complex membrane receptors and Fe-transport systems (Table S3.1). The genome of *Methylogaea oryzae* jcm 16910 contains a *pulE* gene and its homolog *ferE*, which were shown to be involved in Fe(III) reduction in *Geobacter metallireducens* and *Shewanella putrefaciens* (Table S3.2). These findings suggest that *M. oryzae* jcm 16910 and the closely-related methanotrophs

enriched in our microcosms have the genetic potential to use Fe(III) as electron-acceptor for CH_4 oxidation.

Between days 125-220, the Fe(III) reduction extent increased significantly (up to 26%) in the CH_4-amended microcosms (ANOVA, $p<0.005$) (Figure S3.2). More than 1.5 mg Fe(II)/g sediment was produced (Figure 3.3A) and 26 mg/L Fe^{2+} was released into solution (Figure 3.3b). No or only minor (2%) Fe(III) reduction was observed in abiotic CH_4-amended and biotic non-CH_4-amended microcosms (Figure S3.2), confirming that in active CH_4-amended microcosms, Fe(III) was reduced microbially with CH_4 as electron donor. Dissolved As increased significantly (ANOVA, $p<0.005$) to ~3 µg/L (4% of total As). Considering the lower water/sediment ratio in the aquifer (1:8 wt/wt) and porosity of 0.25 (with a sediment density equal to quartz) (Farhana S. Islam et al., 2004) compared to our microcosms (water/sediment = 3.3:1 wt/wt and porosity of 1), the As concentration in our experiment (3 µg/L) corresponds to 80 µg/L in the field. This suggests that in wells with abundant CH_4 (61-509 µg As/L, Figure 3.1b), substantial amounts of As could be released as a consequence of CH_4 oxidation. The calculated CH_4 oxidation rate ($3.16x10^{-6}$ mol CH_4 cm^{-3} yr^{-1}) (Box S3.2) is comparable to those measured in other environments (Table S3.3).

Sequences related to *M. oryzae* that were abundant after 125 days (>30%), decreased in two out of three microcosms after 220 days (to only 0.15 and 1.5% of the total microbial community). Only in one microcosm, *M. oryzae* related sequences represented still ~30%. However, in all three CH_4-amended microbially-active microcosms, another taxon, classified as an archaeon affiliating to *Candidatus* Methanoperedens, became dominant. While its relative abundance before the incubation was only 0.16% and after 125 days it was detected only in one CH_4-amended microcosm (0.3%), after 220 days its relative abundance increased to 41-64% in all three CH_4-amended microcosms. An increase in archaea was also confirmed by qPCR (Figure 3.4a): while bacterial 16S rRNA gene copy numbers, that dominated at day 125, decreased, the archaeal gene copy numbers increased at day 220 by 2 orders of magnitude compared to bacteria and 4 orders of magnitude compared to the initial archaeal population. Furthermore, the *mcrA* gene abundance also increased in all CH_4-amended biotic microcosms correlating with the archaeal 16S rRNA gene copy number and implying that the majority of archaea in our microcosms are capable of CH_4 oxidation. In contrast, *pmoA* gene copy numbers decreased, mirroring the disappearance of *M. oryzae* carrying the *pmoA* gene. RNA-based sequencing showed that *Ca.* Methanoperedens was also the most abundant taxon among the active microbial community at day 220 (Figure 3.4c) suggesting that it overgrew the initially

dominating bacteria related to *M. oryzae* and that *Ca.* Methanoperedens was responsible for the increased Fe(III) reduction between days 125-220.

The rather low abundance (1-4%) of the known Fe(III)-respiring microorganisms *Geobacter* sp. and *Geothrix* sp. (Figure 3.4b) suggests that they were only marginally involved in Fe(III) reduction. The experiments were carried out under anoxic conditions, had no nitrate present and accumulation of dissolved Mn^{2+} and S-species was not observed to a significant extent (Figure S3.4), which ruled out O_2, nitrate, Mn(IV) and oxidized S-compounds as electron acceptors.

A co-occurrence of high As, Fe and CH_4 concentrations was reported for many regions of Southeast Asia. Analysis of >900 groundwater samples across Bengal-, Mekong- and Red River deltas revealed the highest As concentration in methanogenic zones (Buschmann & Berg, 2009) as well as at our field site (Figure 3.1). The relationship between high CH_4, Fe and As was usually explained by degradation of organic material via fermentation and methanogenesis creating reducing conditions that lead to reductive dissolution of As rich-Fe(III)-bearing sediments. Our results demonstrated that CH_4 is omnipresent and can serve as electron-donor for methanotrophic microorganisms promoting Fe(III)-mineral reduction and dissolution and in consequence contribute to mobilization of As bound to these minerals. In summary, our data suggest that CH_4-driven As-mobilization can happen in many environments similar to the studied aquifers in Vietnam, where As-bearing Fe(III) minerals are available and CH_4 is present.

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3.3 Supporting Information

3.3.1 Materials and Methods

Study area and sample collection. The sampling site is situated close to Van Phuc village, about 15 km SE from Hanoi, on a meander of the Red River (20°55'18.7"N, 105°53'37.9"E) (Fig 1). The lithology, geology, mineralogy, characterization and distribution of organic carbon were described previously (Berg et al., 2008; Eiche et al., 2008, 2017; van Geen et al., 2013; Weinman, 2010). Briefly, the North-Western part is characterized by Pleistocene aquifer sands and the groundwater is slightly reduced with As concentrations below the WHO guideline (10 µg/L), whereas the South-Eastern part is of strongly reduced younger grey Holocene sands and the groundwater exceeds the 10 µg/L limit by factors of 10-50 (Eiche et al., 2008). The transition between the contaminated and uncontaminated zones is characterized by changing redox conditions (redox transition zone) where elevated concentrations of CH_4 of up to 45 mg/L were reported (Stopelli et al., 2020).

In October 2017, a first sampling campaign took place during which we collected a sediment core (ø10 cm; each individual piece ca. 1 m long) from 3.3 to 46.3 m below ground surface at the redox transition zone using rotary drilling. Samples from several depths along the core were collected for DNA extraction. Groundwater was collected from 5 wells located in the vicinity of the redox transition zone. Up to 5 L of water pumped directly from the wells was filtered sequentially through 0.8, 0.45 and 0.22 µm pore size filters using filtration holders (Sartorius™ Polycarbonate Filtration Holders). Sediment and filters were kept on dry ice until transported to the laboratory where they were immediately frozen.

In November 2018, a second sampling campaign took place during which another rotary drilling was performed at the same location (3 m away from the 2017 drilling location). For the microcosm setups we chose orange sediments from 40 m depth. Our preliminary data showed that these sediments had high As and Fe contents, and they were the most homogenous regarding lithology and color (which allowed for sufficient quantities of the representative material for all parallel microcosms). Moreover, these sediments are expected to be responsible for the As release observed at that field site. The sediment was stored anoxically (under N_2 atmosphere) at 4°C in the dark and used immediately after shipping to Germany (ca. 1 week). The total Fe, As and Mn contents of the sediment were determined by XRF (Bruker, AXS S4 Explorer). The total S and C contents were quantified by a Carbon-Sulphur-Analyzer (CSA 5003, Leybold Heraeus, Germany) and inorganic carbon (TIC) was determined by Carbon-

Water-Analyzer (CWA 5003, Leybold Heraeus, Germany). The organic carbon content (TOC) was calculated by subtracting inorganic carbon from total C.

The total dissolved gas pressure (TGGP) was measured directly in the field using miniRUEDI, a portable mass spectrometer (MS) able to quantify dissolved gas concentrations in water via a continuous flow of the water sample through a membrane contractor. The membrane allows for separation of the gas components in the (ground)water into a surrounding pre-evacuated headspace, where the total dissolved gas pressure (TDGP) is measured prior to separation and measurement of the individual gases. Complete details about the functionality of this instrument can be found in (Brennwald et al., 2016). In this campaign, the noble gases He, Ar, Kr, in addition to reactive gases N_2, CH_4, O_2 and CO_2 were measured using the miniRUEDI. Calibration for CH_4, and CO_2 was achieved by using a pre-mixed gas bag containing known quantities of CH_4 (1%) and CO_2 (1%) in addition to N_2 (97%) and H_2 (1%).

Microcosm Setup. Semi-sacrificial microcosms were set up by mixing 30 g of sediment from 40 m depth (orange sandy Fe- and As-bearing sediments that were suggested to be susceptible to As mobilization (Figure S3.1) with 100 mL sterile synthetic groundwater medium (modified from Rathi et al. (Rathi et al., 2017); without As and Fe in the medium) in glass serum bottles (total volume 250 mL). Prior to the preparation of the microcosms, the pH of the medium was adjusted to 7.3 by bubbling with CO_2. The pH was monitored along the experiment and it stayed in the range of 7.2-7.8. All microcosms were prepared in an anoxic glovebox (100% N_2), closed with rubber stoppers and aluminum caps. Three different microcosm treatments were prepared: 1) abiotic control (CON-/CH_4), i.e. microbial respiratory processes inhibited by amendment with 160 mM sodium azide (NaN_3) and headspace exchanged with CH_4/CO_2 (ratio of 9:1 under the pressure of 1.5 bar); 2) biotic control (CON+), i.e. microbially active but without any amendments and headspace exchanged with N_2/CO_2 mixture (ratio of 9:1); 3) biotic, i.e. microbially active with the headspace exchanged with CH_4/CO_2 (ratio of 9:1; under the pressure of 1.5 bar). It has to be noted that the amount of added CH_4 was estimated based on the highest observed field concentration. In order to obtain the desired concentration of CH_4 in the liquid phase, the amount of necessary volume of the CH_4 gas in the headspace was calculated based on Henry's law. At first, vials were flushed with CH_4 for 10 min and pressure equalized to 1 bar. Afterwards, 1 part of the headspace was withdrawn and replaced with CO_2 which gave 9:1 CH_4/CO_2 under 1 bar pressure. Subsequently, half of the initial CH_4 and CO_2 volume was injected which resulted in mix of CH_4/CO_2 in the ratio of 9:1 under 1.5 bar pressure (measured with portable monometer). With this condition concentration of dissolved CH_4 should represent ≈ 45 mg/L. The microcosms were kept at 26°C in the dark until analysis (without shaking). At

two time points (day 125 and 220), three bottles of each treatment were sacrificed for geochemical analysis and molecular studies.

Geochemical Analysis. At each time point (day 0, 8, 35, 50, 65, 80, 95, 110, 125, 165, 180, 195, 210, 221), 2 mL of slurry and sediment were withdrawn using the syringe and needle (ø 1.20 x 40 mm) under anoxic conditions. Samples were centrifuged at 14,000 rpm for 5 min. 100 µL of the supernatant were stabilized in 1M HCl (to avoid oxidation of Fe(II)) and diluted with HCl if necessary for Fe(II) quantification using the Ferrozine assay. Depending on the Fe concentration the samples were diluted either in 400 or 900 µL of 1 M HCl, resulting in a final HCl concentration of 0.2 or 0.1 M). One mL of the supernatant was filtered (0.22 µm, PTFE membrane) and stabilized in 1% HNO_3 for As and other elements analysis by ICP-MS (8900, Agilent Technologies, USA). Sediment (0.14±0.03 g wet weight) obtained after centrifugation was digested for 1 h with 1 mL of 6 M HCl. One mL of the digests was centrifuged (5 min, 14000 rpm) and 100 µL of the supernatant was diluted in 1 M HCl. Fe(II) was quantified in triplicate using the Ferrozine Assay (Schaedler et al., 2018). Statistical differences in As and Fe concentration in the different microcosm setups were analyzed with single factor ANOVA while those at selected time points between pairs of treatments were determined using the Student's t-test.

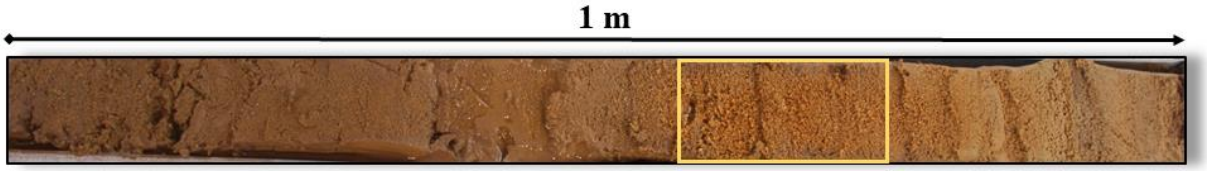
Microbial Community Analysis and Quantitative PCR. The DNA from sediment and groundwater samples collected during 2017 sampling campaign as well as the microcosms sediments samples that were collected at the beginning of the experiment, after 10 days (when maximum Fe(III) reduction and As release were observed) and at the end of the experiment (100 days) was extracted following a phenol-chloroform protocol from Lueders et al. (Lueders et al., 2004). The RNA was successful obtained and transcribed for 3 samples supplied with CH4 (CH4/2, CH4/3, CH4/5). In these samples DNA was digested using TURBO DNA-*free*™ Kit, screening PCR and subsequent gel electrophoresis was performed in order to confirm complete removal of DNA. Afterwards, reverse transcription was performed using SuperScript™ III Reverse Transcriptase. Bacterial and archaeal 16S rRNA genes were amplified from DNA and cDNA using universal primers 515f: GTGYCAGCMGCCGCGGTAA (Parada et al., 2016) and 806r: GGACTACNVGGGTWTCTAAT (Apprill et al., 2015) fused to Illumina adapters. Subsequent library preparation steps (Nextera, Illumina) and 250 bp paired-end sequencing with MiSeq (Illumina, San Diego, CA, USA) using v2 chemistry were performed by Microsynth AG (Switzerland) and between 45,000 and 242,000 read pairs were obtained for each sample.

16S rRNA (gene) sequence analysis. Sequencing data were analyzed with nf-core/ampliseq v1.1.0 that wraps all analysis steps and software and is publicly available at https://github.com/nf-core/ampliseq (Straub et al., 2019). Briefly, primers were trimmed and untrimmed sequences were discarded (<4%) with Cutadapt v1.16 (Martin, 2011). Adapter and primer-free sequences were imported into QIIME2 v2018.06 (Bolyen et al., 2018), quality checked with demux (https://github.com/qiime2/q2-demux), and processed with DADA2 v 1.6.0 (Callahan et al., 2016) to remove PhiX contamination, trim reads (before median quality drops below 35, that is forward 194, reverse 174), correct errors, merge read pairs and remove PCR chimeras and, ultimately, 6,546 amplicon sequencing variants (ASVs) were obtained for groundwater, 2,242 for core sediments from 2017 and 4,544 from microcosms sediments. Alpha rarefaction curves were produced with the QIIME2 diversity alpha-rarefaction plugin which indicated that the richness of the samples has been fully observed. A Naive Bayes classifier was fitted with 16S rRNA gene sequences extracted from SILVA v132 (Pruesse et al., 2007) QIIME compatible database 99% identity clustered sequences using the PCR primer sequences. ASVs were classified by taxon using the fitted classifier (https://github.com/qiime2/q2-feature-classifier). ASVs classified as chloroplast or mitochondria were removed. The number of removed ASVs was 70, 9 and 33 for groundwater, core sediment and microcosm sediments, respectively, totaling to <1% relative abundance per sample and the remaining ASVs had their abundances extracted by feature-table (Pruesse et al., 2007).

Raw sequencing data have been deposited at DDBJ/ENA/GenBank under
BioProject accession number PRJNA593718
(https://www.ncbi.nlm.nih.gov/bioproject/PRJNA593718).

Quantitative PCR. The qPCR specific for the 16S rRNA (genes) of bacteria and archaea as well as for methyl-coenzyme M reductase subunit alpha (*mcrA*) and particulate methane monooxygenase (*pmoA*) genes were performed. The qPCR primer sequences, gene-specific plasmid standards, and details of the thermal programs are given in the Table S3.4. Total numbers of bacteria and archaea genes were estimated by quantitative PCR (qPCR) (Bio-Rad Laboratories GmbH, Munich, Germany) based on the amplification of the 16S rRNA genes. Quantitative PCRs on DNA extracts obtained as described above, were performed in triplicates using SybrGreen® Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on the C1000 Touch thermal cycler (CFX96^{TM} real time system). Plasmid vectors (pCR2.1®, Invitrogen, Darmstadt, Germany) containing a cloned 16S rRNA gene fragment from *Thiomonas* sp. and *Halobacterium salinarum* were used as standards for the quantification of total bacteria and

archaea, respectively. Each qPCR assay was repeated three times with triplicate measurements of each sample per run. Data analysis was done using the Bio-Rad CFX Maestro 1.1, software, version 4.1 (Bio-Rad, 2017). Due to low concentrations of extractable DNA in CON+ and CON- sediments after 125 and 220 days, quantification of bacterial and archaeal 16S rRNA genes as well as *mcrA* and *pmoA* genes was not successful for these samples.



Depth	Fe	Mn	As	C	S
m	%	%	mg/kg	%	%
39.7	0.88	0.004	2.3	0.022	0.004

Figure S3.1 Sediment core obtained from the rotary drilling campaign in 2018 (length of this core is 1 m; it stems from a depth of 39-40 m). The sediment indicated by the yellow box was used for the microcosms. The main properties of this sediment are summarized in the table underneath the photo.

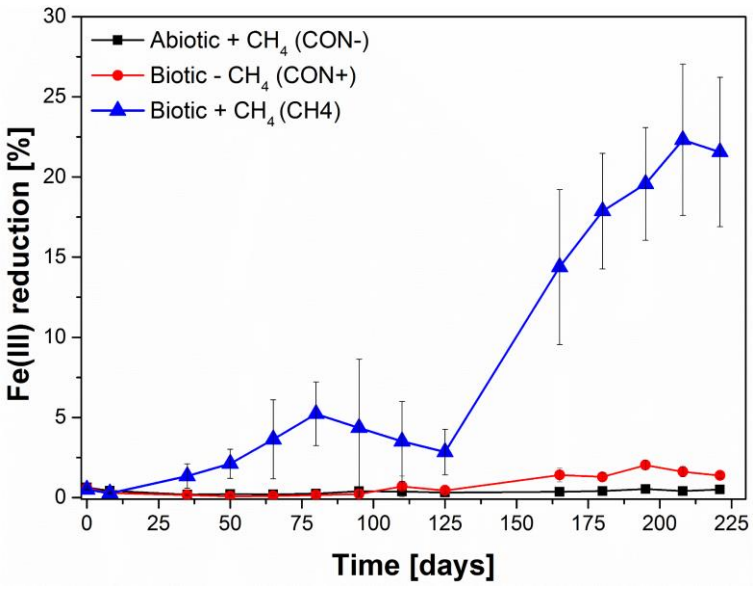


Figure S3.2 Extent of Fe(III) reduction within 220 days of incubation calculated in % based on total Fe present in sediments obtained from XRF. Abiotic control supplied with 160 mM NaN_3 in order to inhibit microbial activity and amended with CH_4 (CON-/CH4), biotically active control without addition of CH_4 (CON+), biotically active microcosms amended with CH_4. Error bars represent standard deviation from 6 bottles until 125 days and 3 bottles from 125 to 220 days. Each bottle was measured in triplicate.

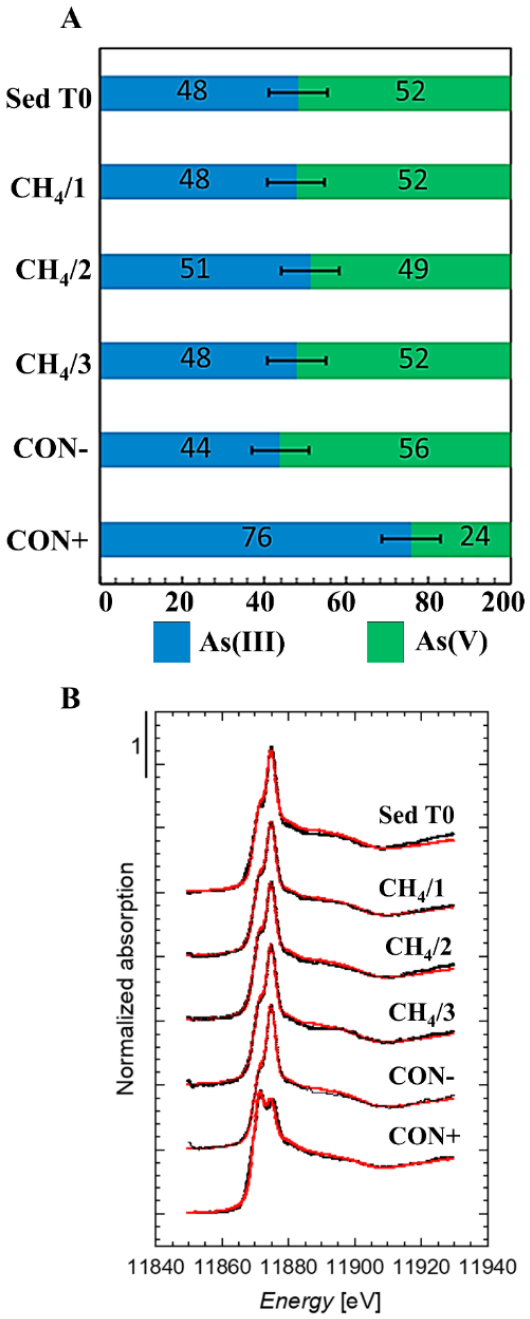


Figure S3.3 Solid-phase As speciation by synchrotron-based X-ray absorption near-edge structure (XANES) spectroscopy. A) proportions of arsenate and arsenite in the sediments before incubation (Sed T0), sediments incubated with CH_4 for 125 days (CH_4/1, CH_4/2, CH_4/3), abiotic control with CH_4 and NaN_3 (CON-), and biotic control without CH_4 fitted in the linear combination fitting (LCF) analysis. B) Normalized As K-edge XANES spectra of microcosms sediments. Please note that there is no significant change in solid-phase As speciation in the abiotic control and in the CH_4-amended microbially active setups. However, in the biotic control, i.e. the setup where microorganisms were active but no CH_4 was added, significant reduction of As(V) to As(III) took place in the solids. Our molecular data (Figure 3.4) suggests that mainly *Geobacter* sp. were responsible for this reduction although the identity of the electron donor remains unknown.

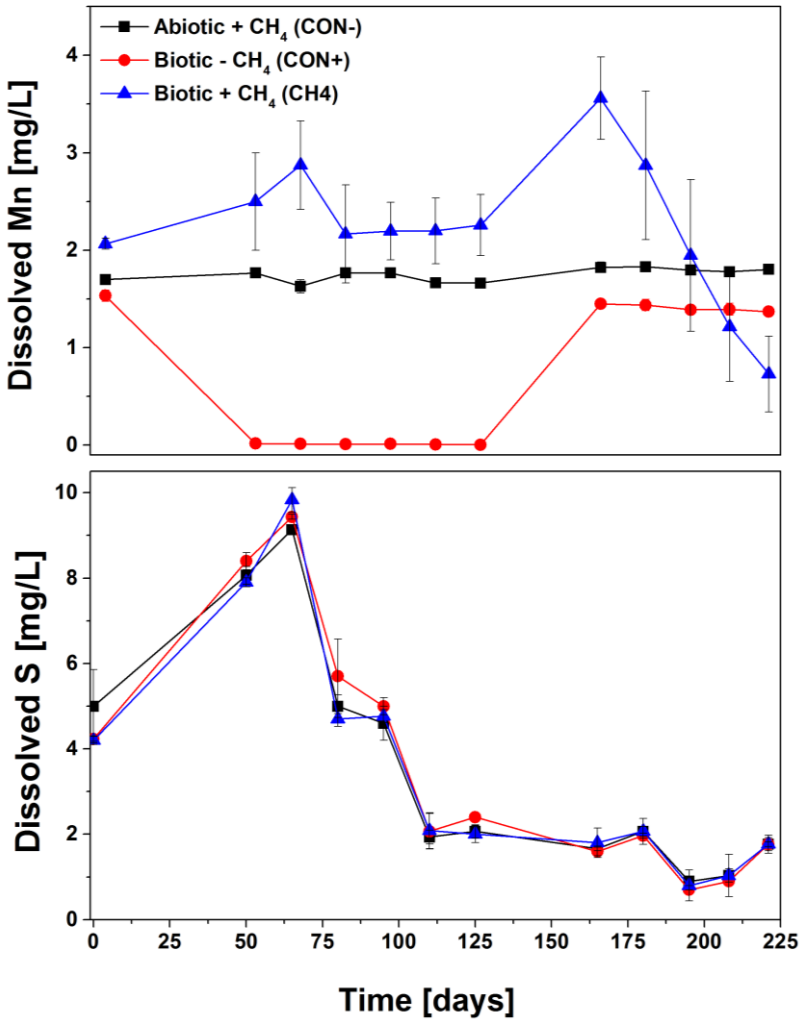


Figure S3.4 Changes in dissolved Mn and S over time. The absence of a significant increase in concentration of Mn and S suggests that Mn(IV) and SO_4^{2-} did not serve as electron acceptors to a significant extent for anaerobic CH_4 oxidation and therefore mainly Fe(III) was used as electron acceptor in our anoxic microcosm setups.

Total Fe/microcosm
XRF → 8.8 mg/g x 30g = 264 mg/microcosm / 55.85 mg/mmol = **4.73 mmol Fe**
Total CH_4/microcosm
Headspace V = 137 mL → CH_4/CO_2 (9:1)
Molar volume of ideal gas at T = 299.15 K and P = 1.5 bar (=150,000 Pa)

$$Vm = \frac{RT}{P} = \frac{8.3145\,\frac{L.kPa}{K.mol} \times 299.15\ K}{150\ kPa} = 16.582\,\frac{L}{mol}$$

CH_4 calculation
Volume of CH_4 in headspace $\frac{123.3\ mL\ CH4 \times 1.5\ bar}{1.0\ bar}$ = 184.95 ml CH_4
Moles of CH_4 = 0.18495 L CH_4 / 16.582 L/mol = **11.154 mmol CH_4 (in headspace)**
CO_2 calculation
Volume of CO_2 in headspace = 13.7 mL CO_2 x 1.5 bar = 20.55 ml CO_2
Moles of CO_2 = 0.02055 L CO_2 / 16.582 L/mol = **1.239 mmol CO_2 (in headspace)**

$CH_4 + 2H_2O \rightarrow CO_2 + 8H^+ + 8e^-$
Reaction stoichiometry → 1 mol of CH_4 will produce 1 mol of CO_2
$Fe^{3+} + e^- \rightarrow Fe^{2+}$
Combined reaction:
$CH_4 + 8Fe^{3+} + 2H_2O \rightarrow CO_2 + 8Fe^{2+} + 8H^+$
Reaction stoichiometry → 1 mol of CH_4 will reduce 8 mol of Fe^{3+}

Therefore, 4.73 mmol of Fe^{3+} can be reduced by 4.73/11.154 = 0.424 mmol of CH_4
Actual amount of CH_4 present in a microcosm = 11.154 mmol
which is 11.154/0.424 = **26.31** times higher than required to reduce 100% Fe(III) in sediment (assuming all Fe is present as Fe(III))

Maximum amount of Fe(III) reduced in microcosm: 26.2% = 2.3 mg Fe/g sediments
2.3 mgFe x 30 g / 55.85 mg/mmol = 1.235 mmol Fe

Amount of CH_4 that is needed to reduce 2.3 mg Fe(III)/g of sediment:
1.235/4.73*0.424 = **0.111 mmol CH_4**
which is 0.111/11.154 = **0.992% of total CH_4 available**
or, this is also 0.111/1.61 = 6.90% of dissolved CH_4

Amount of CO_2 produced from oxidation of 0.111 mmol CH_4:
$CH_4 + 2H_2O \rightarrow CO_2 + 8H^+ + 8e^-$
reaction stoichiometry → 1 mol of dissolved CH_4 will produce 1 mol of dissolved CO_2
or, 0.111 mmol dissolved CH_4 will produce **0.111 mmol dissolved CO_2**

Percentage of CO_2 produced from CH_4 oxidation compared to CO_2 contributed by the bicarbonate buffer
(Calculated based on the PhreeqC model)

Total CO_2 in buffered system = 1.085 mmol (0.158 in headspace + 0.927 dissolved)
Total CO_2 produced from CH_4 oxidation = 0.111 mmol
Therefore, ratio = 0.111/1.085 = 0.102 = **10.2%**

Box S3.1 Calculation showing excess of CH_4 used in microcosms. The high concentration of CH_4 prevented us from seeing the small amount of CH_4 oxidized that was used to reduce the maximum amount of Fe(III) recorded in the sediments. The quantification of CO_2 production over time was also not possible due to the bicarbonate buffer and CO_2 present in the headspace that was much higher than the amount of CO_2 produced from CH_4 oxidation.

Fe(III) reduction time
From day 125 to day 220 → 95 days

Fe(II) already present in sediments at the day 125
0.25 mg/g sediment

Total Fe(II) produced during 95 days (from 125 to 220 days) in sediments
1.76 - 0.25 = 1.51 mg/g sediment

Total Fe(II) produced per 1 microcosm (30g sediments) during 95 days
1.51 x 30 = 45.3 mg Fe(II)/30g sediments

Total Fe^{2+} released in 95 days
24.7 mg/L

Total Fe^{2+} released in 95 days per 1 microcosm (100 mL)
24.7 x 0.1 = 2.47 mg/0.1L

Total Fe(II) produced per microcosm during 95 days
45.3 + 2.47 = 47.8 mg/ 55.8 = 0.85 mmol Fe(II)

Volume of microcosms (sediments + water)
130 cm^3

mmol of Fe(II) produced per 1 day
0.85 mmol Fe(II)/95 days = 0.009 mmolFe(II)/day

mmol of Fe(II) produced per 1 day per microcosm
0.009/130 = 6.9E-05 mmolFe(II)/day/cm^3

Amount of CH_4 oxidized per day
$\mathbf{CH_4 + 8Fe^{3+} + 2H_2O \rightarrow CO_2 + 8Fe^{2+} + 8H^+}$
Reaction stoichiometry → 1 mol of CH_4 will reduce 8 mol of Fe^{3+}
6.9E-05/8 = 8.7E-06 [mmol CH_4/day/cm^3]

Amount of CH_4 oxidized per year
8.7E-06 x 365 = 0.00316/1000 = 3.16E-06 [mmol CH_4/cm^3/year]

Box S3.2 Calculation showing anaerobic CH_4 oxidation based on the amount of Fe(III) reduced during 95 day of incubation (between 125 and 220 days).

Table S3.1 Protein coding genes involved in cytochrome c functioning in *Methylogaea oryzae* jcm 16910 based on the complete genome available on IMG/MER (ID: 2675903437).

GENE ID	LOCUS TAG	GENE PRODUCT NAME
2677966701	Ga0128369_101124	**cytochrome c** peroxidase
2677970667	Ga0128369_14851	
2677969450	Ga0128369_12346	
2677966982	Ga0128369_10214	**cytochrome c** oxidase assembly protein subunit 15
2677967072	Ga0128369_10251	**cytochrome c**-type biogenesis protein CcmF
2677967073	Ga0128369_10252	
2677967074	Ga0128369_10253	
2677967075	Ga0128369_10254	
2677967677	Ga0128369_10592	ubiquinol-**cytochrome c** reductase iron-sulfur subunit
2677967678	Ga0128369_10593	ubiquinol-**cytochrome c** reductase cytochrome b subunit
2677967680	Ga0128369_10595	ubiquinol-**cytochrome c** reductase **cytochrome c**1 subunit
2677968180	Ga0128369_109310	alcohol dehydrogenase (**cytochrome c**)
2677968405	Ga0128369_11125	**cytochrome c** oxidase subunit 1
2677970933	Ga0128369_15702	
2677970934	Ga0128369_15703	
2677971172	Ga0128369_16554	
2677968404	Ga0128369_11124	**cytochrome c** oxidase subunit 2
2677971171	Ga0128369_16553	
2677968408	Ga0128369_11128	**cytochrome c** oxidase subunit 3
2677968407	Ga0128369_11127	**cytochrome c** oxidase assembly protein subunit 11
2677968527	Ga0128369_112211	**cytochrome c**
2677968540	Ga0128369_11242	
2677970935	Ga0128369_15704	
2677968995	Ga0128369_11716	**Cytochrome c**553
2677970151	Ga0128369_13602	
2677969011	Ga0128369_11737	**Cytochrome C** oxidase, cbb3-type, subunit III
2677971138	Ga0128369_16413	
2677969052	Ga0128369_11782	methanol dehydrogenase (**cytochrome c**) subunit 1
2677969056	Ga0128369_11786	methanol dehydrogenase (**cytochrome c**) subunit 2
2677969054	Ga0128369_11784	**cytochrome c**-L
2677969055	Ga0128369_11785	
2677969170	Ga0128369_11921	**cytochrome c**-type biogenesis protein CcmH
2677970206	Ga0128369_13715	
2677969171	Ga0128369_11922	**cytochrome c** biogenesis protein CcmG
2677966370	Ga0128369_10016	**Iron** complex outer membrane receptor protein
2677967094	Ga0128369_10261	
2677967095	Ga0128369_10262	
2677967248	Ga0128369_10346	
2677967249	Ga0128369_10347	
2677967491	Ga0128369_104620	
2677968384	Ga0128369_11103	
2677969233	Ga0128369_12004	
2677970108	Ga0128369_13521	
2677970109	Ga0128369_13522	
2677970771	Ga0128369_15162	
2677971405	Ga0128369_17581	
2677966404	Ga0128369_10021	**Iron(III)** transport system permease protein
2677967816	Ga0128369_10681	
2677966405	Ga0128369_10022	**Iron(III)** transport system substrate-binding protein
2677966406	Ga0128369_10023	
2677967817	Ga0128369_10682	**Iron(III)** transport system ATP-binding protein
2677967818	Ga0128369_10683	
2677970101	Ga0128369_13504	Uncharacterized **iron**-regulated membrane protein

Table S3.2 Genes commonly found in Fe(III) reducing bacteria present in *Methylogaea oryzae* jcm 16910 genome (IMG/MER, ID: 2675903437). Presence of these genes suggests that *M. oryzae* jcm 16910 is genetically equipped to use Fe(III) as electron acceptor. Low identity compared to source genes is due to fact that these genes are not conservative.

Gene	Putative Function	Source	Identity %	Reference
***pul*E**	Type II secretion system ATPase. Required to respire anaerobically on Fe(III) or Mn(IV).	*Geobacter metallireducens*	44	DiChristina et al. 2002
***fer*E**	Protein secretion by the type II secretion system. Required to respire anaerobically on Fe(III) or Mn(IV). Homolog to *pul*E	*Shewanella putrefaciens*	34	DiChristina et al. 2002

Table S3.3 Fe-dependent anaerobic oxidation of methane rates in different environments. Modified from Aromokeye et al., 2020.

Site	Fe-AOM rates (mol CH_4 cm^{-3} yr^{-1})	References
Vietnam aquifer	2.06E-06	This study
North Sea	3.47E-08	Aromokeye et al., 2020
Baltic Sea	1.10E-09	Egger et al., 2017
Black Sea	1.46E-11	Egger et al., 2016
Bothnian Sea	1.30E-06	Egger et al., 2015
Eel River Basin seep	6.00E-06	Beal et al., 2009
Chowder Hill hydrothermal vent	5.90E-05	Wankel et al., 2012

Table S3.4 List of primers, primers sequences and thermal programs used for quantification of bacterial and archaeal 16S rRNA gene, particulate methane monooxygenase (*pmoA*) and methyl-coenzyme M reductase subunit alpha (*mcrA*) gene copy numbers.

Specificity	Primer	Primer sequence (5′→ 3′)	Thermal program	References
16S rRNA gene *Bacteria*	341f	CCT ACG GGA GGC AGC AG	98°C - 2′; (98°C - 5′′; 60°C - 12′′	Muyzer et al., 1993
	534r	ATT ACC GCG GCT GCT GG	95°C - 1′; 60°C - 1′) x 40; 60 – 95°C - 10′′	
16S rRNA gene *Archaea*	Ar109f	ACK GCT GAG TAA CAC GT	98°C - 3′; (98°C - 5′′; 52°C - 12′′	Großkopf et al., 1998 Stahl and Amann 1991
	Ar915r	GTG CTC CCC CGC CAA TTC CT	72°C - 15′) x 40; 98°C - 1′; 52°C - 1′; 52 – 95°C - 10′′	
pmoA	A189f A682r	GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASG C	96°C - 5′; (94°C - 1′; 56°C - 1′; 72°C - 1′) x 38; 72°C - 5′	Holmes et al. 1995
*mcr*A	ME1f ME1r	GCMATGCARATHGGWAT GTC TCATKGCRTAGTTDGGRT AGT	95°C - 5′; (95°C - 50′′; 54°C - 50′′ 72°C - 50′) x 34; 72°C - 10′	Hales et al. 1996

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Chapter 4 – personal contributions

The sampling campaigned in Vietnam was coordinated by Prof. Michael Berg and jointly performed by all AdvectAs project members. The groundwater samples for molecular studies were collected, processed and analyzed by myself while samples for hydrogeochemical studies were collected, processed and analyzed by Dr. Emiliano Stopelli. Therefore Dr. Emiliano Stopelli and myself are equally contributing authors of this work. The hypothesis was formulated by myself and discussed with Dr. Emiliano Stopelli, Jun.-Prof. Sara Kleindienst, Prof. Michael Berg and Prof. Andreas Kappler. The bioinformatics treatment of 16S rRNA gene sequences was performed by Dr. Daniel Starub. The manuscript was written by myself with the support of Dr. Emiliano Stopelli and revised by all co-authors.

Chapter 4: Arsenic behavior in groundwater in Hanoi (Vietnam) influenced by a complex biogeochemical network of iron, methane, and sulfur cycling

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4.1 Abstract

The fate of arsenic (As) in groundwater is determined by multiple interrelated microbial and abiotic processes that contribute to As (im)mobilization. Most studies to date have investigated individual processes related to As (im)mobilization rather than the complex networks present *in situ*. In this study, we used RNA-based microbial community analysis in combination with groundwater hydrogeochemical measurements to elucidate the behavior of As along a 2 km transect near Hanoi, Vietnam. The transect stretches from the riverbank across a strongly reducing and As-contaminated Holocene aquifer, followed by a redox transition zone (RTZ) and a Pleistocene aquifer, at which As concentrations are low. Our analyses revealed fermentation and methanogenesis as important processes providing electron donors, fueling the microbially mediated reductive dissolution of As-bearing Fe(III) minerals and ultimately promoting As mobilization. As a consequence of high CH_4 concentrations, methanotrophs thrive across the Holocene aquifer and the redox transition zone. Finally, our results underline the role of SO_4^{2-}-reducing and putative Fe(II)-/As(III)-oxidizing bacteria as a sink for As, particularly at the RTZ. Overall, our results suggest that a complex network of microbial and biogeochemical processes has to be considered to better understand the biogeochemical behavior of As in groundwater.

4.2 Introduction

Arsenic (As) groundwater contamination has been extensively studied for over two decades, and our knowledge about its mobility and behavior in the environment has increased substantially in the past years. Arsenic-bearing sediments deposited in the river delta regions of South and Southeast Asia have been of particular interest (Acharyya et al., 2000; Berg et al., 2007; Dowling et al., 2002; Postma et al., 2007). Reducing conditions in these aquifers led to the mobilization and enrichment of As in groundwater (Charlet & Polya, 2006; Smedley & Kinniburgh, 2002). Moreover, these regions are among the most densely populated areas on the planet (Smedley & Kinniburgh, 2002; Smith et al., 2000), and many of their inhabitants, mainly situated in rural areas, still rely on water from shallow wells that is then used either untreated or filtered through simple sand filters (Berg et al., 2006; Nitzsche et al., 2015). Because As is “invisible”, it does not affect the taste or smell of water and food, and, thus, many people have been unconsciously exposed to this toxic metalloid for years. For this reason, chronical exposure to As has led to massive poisoning throughout local communities, manifesting as

dermal lesions, cardiovascular diseases, and various types of cancer (Karagas et al., 2015; Smith A H et al., 1992).

Many mechanisms for the release of As into groundwater have been proposed (Farhana S. Islam et al., 2004), yet the most accepted is that As-bearing Fe(III) (oxyhydr)oxide minerals are reduced and thus dissolved by microorganisms, a process that is coupled with the oxidation of organic carbon (Chatain et al., 2005; F. S. Islam, Pederick, et al., 2005; Farhana S. Islam et al., 2004). A large number of laboratory studies have been conducted in order to underpin microbially mediated Fe(III) mineral reductive dissolution and subsequent As mobilization. These studies have not only revealed the identity of microorganisms driving this process, such as *Geobacter* sp. (F. S. Islam, Pederick, et al., 2005), *Shewanella* (Cummings et al., 1999), or *Geothrix* sp. (F. S. Islam, Boothman, et al., 2005), but also underline the importance of carbon quantity, quality, and bioavailability as necessary fuels for Fe(III) mineral reduction (Chatain et al., 2005; Duan et al., 2008; Glodowska et al., 2020; Héry, M. et al., 2010; Neumann et al., 2014). Nonetheless, additional microbially mediated processes can release As from sediments and contribute to groundwater contamination. For instance, As(V)-reducing bacteria can use As(V) as an electron acceptor and reduce it to the more mobile species of As(III) [15–18]. Amongst others, bacteria belonging to *Geobacter* sp. and *Sulfurospirillum* have been identified to be capable of dissimilatory As(V) reduction (Héry et al., 2008). In addition, isolated from an As-contaminated aquifer, *Enterobacter* (ARS-3) has been shown to release up to 15 µg/L of As from shallow reducing aquifer sediments via the direct enzymatic reduction of As(V) (Liao, Chu, Su, Lin, et al., 2011).

In contrast, a variety of microbial processes was shown to be a sink for As in groundwater in laboratory experiments. Iron(II)-oxidizing bacteria, such as chemoautotrophic *Gallionella* sp. (Hallbeck & Pedersen, 1990) and heterotrophic *Leptothrix* sp. (Hashimoto et al., 2007), were found to catalyze As removal via the precipitation of Fe(III) (oxyhydr)oxides and the sorption of As onto biogenic Fe minerals (Hohmann et al., 2010; Katsoyiannis & Zouboulis, 2006). Depending on the As to Fe ratio, different types of Fe minerals can be produced, which has been specifically shown for nitrate-dependent Fe(II)-oxidizing *Acidovorax* (Hohmann et al., 2011). Interestingly, even if Fe(III) bio-reduction generally leads to As and Fe mobilization into solution, also different secondary minerals can be formed, such as magnetite, which can contribute to the re-sequestration of Fe and As (Muehe et al., 2016). In addition, microbial chemolithoautotrophic or heterotrophic As(III) oxidation can also be a potential sink for dissolved As, since microorganisms mediating this process can transform As(III) into the less

mobile As(V), which is more prone to sorption to Fe(III) (oxyhydr)oxide minerals (Garcia-Dominguez et al., 2008; Ike et al., 2008).

Several studies have been conducted to understand how microbial SO_4^{2-} reduction affects As mobility. On the one hand, SO_4^{2-} reduction can lead to the precipitation of dissolved Fe(II) as iron sulfides along with the incorporation and sorption of As and/or to the direct precipitation of arsenic trisulfide (As_2S_3) (Bostick & Fendorf, 2003; Newman et al., 1997). On the other hand, SO_4^{2-} reduction and sulfide formation can also contribute to a reduction in Fe(III) minerals and subsequently either release some As that was bound to these minerals or directly mobilize As in the form of thioarsenates (Kumar et al., 2020). As a consequence, some studies have shown that SO_4^{2-} reduction leads to As removal (Kirk et al., 2004; Rittle et al., 1995), while others have reported opposite observations, in which As concentrations in the water increased under SO_4^{2-}-reducing conditions (Guo et al., 2016; Kumar et al., 2016; Stucker et al., 2014). The sulfide/Fe molar ratio seems to control this behavior (Kumar et al., 2020). However, a previous study from Red River Delta aquifers showed that SO_4^{2-}-reducing conditions are associated with net As immobilization (Sracek et al., 2018), which is likely due to rather low SO_4^{2-} concentrations and, in consequence, low sulfide/Fe ratios.

In addition to microbial mediated Fe, As, and S redox cycles affecting As (im)mobilization into groundwater, there are additional microbial processes, such as fermentation, methanogenesis, or methanotrophy, that have not been the focus in understanding the fate of As. Wang et al. (Wang et al., 2015) previously suggested an involvement of methanogens in As mobilization. Indeed, in many regions of South and Southeast Asia, the co-occurrence of high concentrations of As, Fe, and CH_4 has been reported (Harvey et al., 2002; Jessen et al., 2008; Liu et al., 2009; Polizzotto et al., 2005; Postma et al., 2007, 2012). For example, in southern Bangladesh, CH_4, driven from the degradation of dissolved inorganic carbon (DIC), reached 1.3 mM (21 mg/L), and, at the same depth, a peak for dissolved As was reported (Harvey et al., 2002). Similar correlations were found across Bengal, Mekong, and Red River deltas, where As concentrations were significantly higher in methanogenic zones yet significantly lower in SO_4^{2-}-reducing and Fe(III)-reducing zones (Buschmann & Berg, 2009). While there seems to be a link between CH_4 and As, this relationship still remains elusive.

Moreover, to date, most studies have focused on a single microbial process under laboratory conditions rather than on the complex network of several processes co-occurring simultaneously in the field. Therefore, here, we used RNA-based 16S rRNA amplicon sequencing for *in situ* active microbial taxa in combination with phylogenetic and functional

gene quantification and hydrogeochemical measurements in order to explain the microbial and biogeochemical processes responsible for the behavior of As in groundwater at our field site. In addition, we predicted the dominating metabolic functions of the *in situ* active microbial community using the 16S rRNA amplicon sequencing data to further corroborate our findings. The field site is located in Van Phuc, about 15 km southeast from the capital city Hanoi, Red River delta region, Vietnam, and it is characterized by zones of distinct hydrogeochemical conditions accompanied by changing groundwater As concentrations. Generally, the southeast part of the aquifer consists of strongly reduced grey Holocene sands and groundwater exceeding the WHO limit of 10 µg/L by a factor of 10-60. The northwest part consists of less reduced orange Pleistocene aquifer sands, and the groundwater presents As concentrations below 10 µg/L (Eiche et al., 2008). The transition between the contaminated and uncontaminated zones is characterized by changing redox conditions (i.e. a redox transition zone), under which As mobilization and immobilization seem to co-occur. A detailed description of the site, including the spatial and temporal evolution of As concentrations in the context of hydrogeochemical conditions, was described previously (Stopelli et al., 2020).

With its heterogeneous conditions, this field site provides ideal conditions for studying the complex microbial and biogeochemical network that affects As mobility in groundwater. Therefore, the objectives of the present study are 1) to identify the main active microbial taxa *in situ*; 2) to correlate active microbial key taxa with hydrogeochemical parameters; and 3) to define the microbial processes and hydrogeochemical conditions affecting the fate of As in groundwater.

4.3 Material and Methods

4.3.1 Study area

The study site is in Vietnam and about 15 km southeast from Hanoi in Van Phuc village, which is situated inside a meander of the Red River (20°55'18.7"N, 105°53'37.9"E). Detailed information about the lithology, mineralogy, geology, and hydrochemistry of the site are available elsewhere (Eiche et al., 2008, 2017; Stopelli et al., 2020; van Geen et al., 2013). An important feature of the site is an inversed groundwater flow towards Hanoi city and is caused by a depression cone due to increased groundwater abstraction in Hanoi. As a result, water flows in a northwest direction with an estimated velocity of 40 m/year (van Geen et al., 2013). Generally, the studied transect can be divided into five main zones (Figure 4.1), as described in Stopelli et al. (Stopelli et al., 2020). Zone A is the riverbank (Red River) in which young

sedimentary deposits are rich in organic matter and As is mobilized (Wallis et al., 2020). Zone B is located near the riverbank in the Holocene aquifer low in dissolved and sedimentary organic carbon (OC), in which Fe(III)- and SO_4^{2-}-reducing conditions are present. In this zone, processes of reductive As dissolution and sorption/incorporation of As into newly formed iron- and sulfur phases co-occur simultaneously and seem to be balanced. Zone C is located further downstream the Holocene aquifer, where, most likely, an input of OC is occurring. In consequence, methanogenic conditions are present, and As enrichment in the groundwater is observed. The redox transition zone at which the advection/intrusion of reduced groundwater from the Holocene aquifer to the Pleistocene aquifer takes place, is defined as zone D. Here, a decrease of As due to sorption on and incorporation into Fe(II) and Fe(II)/Fe(III) minerals is observed, supported by the oxidation of dissolved Fe(II) and the precipitation of Fe(III) minerals. Finally, zone E is situated in the less reducing Pleistocene aquifer, in which As concentrations are below 10 µg/L. In total, 18 wells were analyzed for this study; two wells (AMS 15 and 13) were in the proximity of the transect within comparable hydro(geo)chemical zones and were thus included in the data interpretation (light blue wells in Figure 4.1).

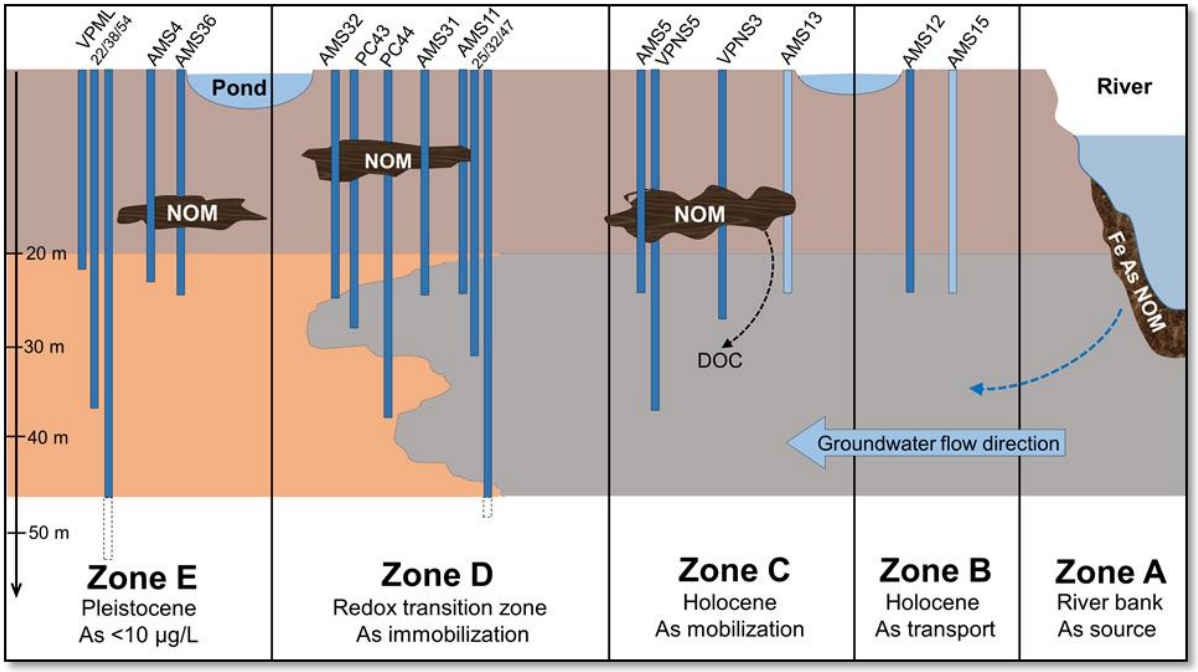


Figure 4.1 Two-dimensional cross-section of Van Phuc aquifers divided by hydrogeochemical zone (A-E), as reported in Stopelli et al. (2020), presenting the distribution of monitoring wells across the transect. Lighter blue wells are in the proximity to the transect within comparable hydrogeochemical zones.

4.3.2 Sample collection and preservation

The groundwater sampling campaign took place in November 2018. Groundwater samples for hydrogeochemical analyses were collected, preserved, and analyzed as described previously in detail (Stopelli et al., 2020). Before sample collection, the groundwater wells were flushed until the stabilization of O_2, and pH and redox potential E_h, were measured using a portable multi-analyzer (WTW 3630). E_h values were normalized to the standard hydrogen electrode (SHE). Trace elements and cations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500 and 8900), anions by ion chromatography (Metrohm 761 Compact IC), dissolved nitrogen (DN) and dissolved organic carbon (DOC) by a total N and C analyzer (Shimadzu TOC-L CSH), and NH_4^+ and ortho-PO_4^{3-} by photometry using the indophenol and molybdate methods, respectively. Alkalinity was determined directly in the field via titration (Merck Alkalinity Test Kit Mcolortest 11109). Methane was analyzed via gas chromatography (Shimadzu GC-2014) using the headspace equilibration method (Sø et al., 2018).

For microbial community analysis, samples were also obtained after the stabilization of O_2, pH, and redox potential E_h (see above). Water was collected in 5 L plastic bottles that were ethanol-sterilized and rinsed with the collected water prior to sampling. Subsequently, the water was immediately filtered through 0.22 µm pore size sterile membrane filters (EMD Millipore) using a suction-type filter holder (Sartorius 16510) connected to a laboratory vacuum pump (Microsart®). In total, 18 wells were sampled, and, from each well, 10 L of water was filtered. The filters were carefully folded and placed into sterile Falcon tubes and immersed in LifeGuard Soil Preservation Solution (Qiagen) in order to stabilize the microbial RNA. Samples were stored on dry ice during transport and placed in a -80 °C freezer upon arrival at the laboratory.

4.3.3 DNA and RNA extraction, DNA digestion, reverse transcription, and amplification

DNA and RNA were extracted using a phenol-chloroform method following a protocol from Lueders et al. (Lueders et al., 2004). RNA and DNA were eluted in 50 µL of a 10 mM Tris buffer. DNA and RNA concentrations were determined using a Qubit® 2.0 Fluorometer with DNA and RNA HS kits (Life Technologies, Carlsbad, CA, USA). Subsequently, RNA extracts were digested with the Ambion Turbo DNA-free™ kit, as directed by the manufacturer (Life Technologies, Carlsbad, CA, USA). Successful DNA removal was confirmed via 30-cycle PCR using general bacterial primers (see below). Afterwards, reverse transcription reactions were performed using a reverse transcriptase (SuperScript™ III), as described by the manufacturer. Bacterial and archaeal 16S rRNA genes were amplified using universal primers 515f: GTGYCAGCMGCCGCGGTAA (Parada et al., 2016) and 806r:

GGACTACNVGGGTWTCTAAT (Apprill et al., 2015) fused to Illumina adapters. The PCR cycling conditions were as follows: 95 ℃ for 3 min, 25 cycles of 95 ℃ for 30s, 55 ℃ for 30 s, and 75 ℃ for 30 s. This was followed by a final elongation step at 72 ℃ for 3 min. The quality and quantity of the purified amplicons were determined using agarose gel electrophoresis and Nanodrop (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). Subsequent library preparation steps and sequencing were performed using Microsynth AG (Balgach, Switzerland). Sequencing was performed on an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA) using the 2 × 250 bp MiSeq Reagent Kit v2 (500 cycles kit), and between 49,771 and 195,960 read pairs were obtained for each sample. Due to insufficient RNA concentrations in the AMS 12 sample, reverse transcription was not successful, and, therefore, we performed only DNA-based analysis for this sample.

4.3.4 16S rRNA (gene) sequence analysis

Sequencing data was analyzed with nf-core/ampliseq v1.0.0, which includes all analysis steps and software and is publicly available (D. Straub et al., 2019). Primers were trimmed, and untrimmed sequences were discarded (< 4%) with Cutadapt version 1.16 (Martin, 2011). Adapter and primer-free sequences were imported into QIIME2 version 2018.06 (Bolyen et al., 2018), their quality was checked with demux (https://github.com/qiime2/q2-demux), and they were processed with DADA2 version 1.6.0 (Callahan et al., 2016) to eliminate PhiX contamination, trim reads (before median quality drops below 35; forward reads were trimmed at 230 bp and reverse reads at 207 bp), correct errors, merge read pairs, and remove PCR chimeras; ultimately, 18,642 amplicon sequencing variants (ASVs) were obtained across all samples. Alpha rarefaction curves were produced with the QIIME2 diversity alpha-rarefaction plugin, which indicated that the richness of the samples had been fully observed. A Naive Bayes classifier was fitted with 16S rRNA (gene) sequences extracted from the SILVA version 132 SSU Ref NR 99 database (Pruesse et al., 2007), using the PCR primer sequences. ASVs were classified by taxon using the fitted classifier (https://github.com/qiime2/q2-feature-classifier). ASVs classified as chloroplasts or mitochondria were removed. The number of removed ASVs was 34, totaling to < 0.1% relative abundance per sample, and the remaining ASVs had their abundances extracted by feature-table (Pruesse et al., 2007). The abundance table was rarefied with a sampling depth of 38,217—the number of minimum counts across samples—and Bray-Curtis dissimilarities were calculated with q2-diversity (https://github.com/qiime2/q2-diversity).

Pathways, i.e. MetaCyc ontology predictions, were inferred with PICRUSt2 version 2.2.0-b (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille

et al., 2013) and MinPath (Minimal set of Pathways) (Ye & Doak, 2009) using ASVs and their abundance counts. Inferring metabolic pathways from 16S rRNA amplicon sequencing data is certainly not as accurate as measuring genes by shotgun metagenomics, but it yields helpful approximations to support hypotheses driven by additional microbiological and biogeochemical analyses (Langille et al., 2013).

The raw sequencing data has been deposited at GenBank under BioProject accession number PRJNA628856 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA628856).

4.3.5 Statistical analysis

All statistical analyses were carried out in R (http://cran.r-project.org/, version 3.4.4, 2018-03-15). The Bray-Curtis dissimilarity (Sorensen et al., 1948), calculated via QIIME2, was plotted using Non-metric Multidimensional Scaling (NMDS) via phyloseq (McMurdie & Holmes, 2013) version 1.22.3. Environmental variables were fitted onto the ordination and denoted by arrows using vegan version 2.5-1 (Oksanen et al., 2019), and the significance of the fitted vectors was assessed using 999 permutations of environmental variables. Spearman rank correlations were determined between the hydrogeochemical parameters and the relative abundance of the dominant taxa, and *p*-values were corrected for multiple testing using the Benjamini-Hochberg method, yielding a false discovery rate (FDR) (Benjamini & Hochberg, 1995).

4.3.6 Quantitative PCR

Quantitative PCRs specific for 16S rRNA genes of bacteria and archaea, methyl-coenzyme M reductase subunit alpha (*mcrA*) genes, particulate methane monooxygenase (*pmoA*) genes, arsenate reductase (*arr*A) genes, and *Geobacter* sp. genes were performed. The qPCR primer sequences, gene-specific plasmid standards, and details on the thermal programs are given in Table S4.1. Quantitative PCRs on DNA extracts obtained as described above were performed in triplicate using SybrGreen® Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on the C1000 Touch thermal cycler (CFX96^{TM} real time system). Each quantitative PCR assay was repeated three times, with triplicate measurements calculated for each sample per run. Data analysis was done using the Bio-Rad CFX Maestro 1.1 software version 4.1 (Bio-Rad, 2017).

4.4 Results and Discussion

The microbial diversity based on 16S rRNA (gene) amplicon sequencing in the groundwater samples correlated significantly with groundwater As (p = 0.03), CH_4 (p = 0.001), NH_4^+ (p = 0.01) and Mn (p = 0.05). This implies that different concentrations of these geochemical species

were responsible for distinct microbial community assemblages among the analyzed wells, or, vice versa, the microbial communities are influencing the fate of these geochemical species in the groundwater (Figure 4.2). Non-metric Multidimensional Scaling (NMDS) resulted in the grouping of the wells, which largely reflected the hydrogeochemical zonation proposed by Stopelli et al. (Stopelli et al., 2020). Therefore, in the forthcoming sections, we follow this zonation and combine hydrogeochemical data with the analysis of active microbial taxa, focusing specifically on processes that affect As mobilization and immobilization *in situ* to explain the behavior of As in each zone.

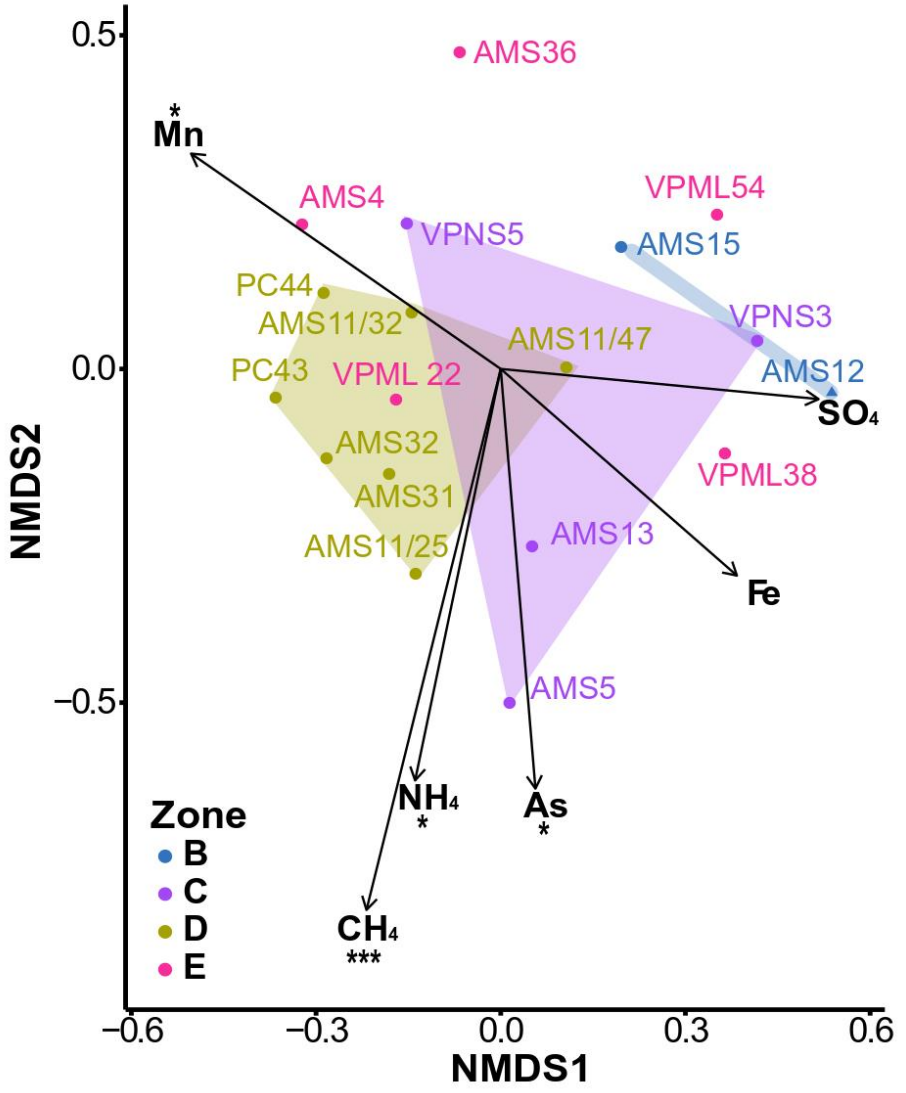


Figure 4.2 Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity (stress = 0.17) to visualize the main biogeochemical groundwater parameters (arrows for As, Fe, CH_4, SO_4^{2-}, NH_4^+, and Mn) associated with the microbial community composition. The strength of the interaction is shown by the length of the arrows; significant correlations are shown for As (*, $p < 0.03$), CH_4 (***, $p < 0.001$), NH_4^+ (*, $p < 0.01$) and Mn (*, $p < 0.05$).

4.4.1 Zone A and B: From riverbank sediments to the Holocene aquifer—As mobilization and transport

River bank sediments (zone A) are a source of dissolved As (10-508 µg/L As in the porewater, inter-annual average of 100-120 µg/L (Stopelli et al., 2020)). This zone was also characterized by elevated concentrations of dissolved organic carbon (DOC) (5.0-6.9 mg C/L) and dissolved Fe (up to 13 mg/L) (Table 4.1). These results are in line with the reactive transport modeling of the Van Phuc aquifers, where the riverbank-aquifer interface has been identified as a biogeochemical reaction hotspot and a source of elevated As concentrations (Stahl et al., 2016; Wallis et al., 2020). This is due to the constant supply of sediments rich in bioavailable C and reactive Fe(III) (oxyhydr)oxides from the Red River, promoting Fe(III)-reducing conditions. Two wells in zone B (AMS12 and AMS15) were located in direct proximity to the Red River in the Holocene aquifer (~200 m downstream of the riverbank; zone B, Figure 4.1). Arsenic (135 and 22 µg/L, respectively) and Fe concentrations (12 and 0.54 mg/l, respectively) in these two wells differed from each other. These differences are likely related to the river bank geomorphology, as AMS15 lies closer to an erosional meander, while AMS12 is located on a depositional one, in which more sediments can be deposited, and, thus, a greater amount of As can subsequently be released (Stahl et al., 2016). However, the DOC concentrations were quite similar in both wells (1.2-1.4 mg/L) and lower than in the riverbank porewater in zone A (5.0-6.9 mg C/L), implying that C consumption was taking place between zones A and B. Wells in zone B present dissolved As concentrations comparable to the average values in the riverbank pore water in zone A (100-120 µg/L), suggesting a net transport of As from the riverbank to the Holocene aquifer. This might also be related to the co-occurrence of microbial activities leading to a net balance between As mobilization and immobilization.

Generally, bacterial 16S rRNA gene copy numbers in zone B (Figure 4.3) appeared to be lower (up to $5.0 \times 10^4 \pm 5.0 \times 10^3$/mL), while archaeal gene copy numbers were relatively high (up to $3.7 \times 10^3 \pm 3.5 \times 10^2$/mL) compared to other zones. The observed 16S rRNA gene amplicon sequencing variants (ASVs) and the alpha diversity indices were highest in zone B compared to all other zones, suggesting that this zone has the greatest microbial diversity (Table S4.2), which might be reflected by diverse microbial processes, which lead to simultaneous As mobilization and immobilization with the observed limited net change in dissolved As concentrations.

Table 4.1 Hydrogeochemical parameters measured in groundwater wells in the Van Phuc aquifer, Vietnam.

Zone		LOQ	Riverbank	B		C				D							E				
Well ID				AMS 12	AMS 15	AMS 5	VPNS 5	VPNS 3	AMS 13	AMS 11(25)	AMS 11(32)	AMS 11(47)	AMS 32	PC 44	AMS 31	PC 43	VPML 22	VPML 38	VPML 54	AMS 36	AMS 4
Depth	m	-	0.2-0.5	23-24	23-24	23-24	35-36	25-26	23-24	23-24	30-31	45-46	23-24	36-37	23-24	26-27	20-21	36-37	52-53	23-24	22-23
pH	-	-	6.42-7.25	6.96	7.04	7.00	7.09	7.22	7.03	7.40	7.09	6.69	7.22	6.96	7.21	7.21	6.74	6.60	6.74	7.04	7.04
O_2	mg/L	-	2.03-7.05	0.03	0.03	0.05	0.03	0.35	0.62**	0.07	0.02	0.08	0.09	0.02	0.05	0.06	0.05	0.04	0.05	0.08	0.03
E_h (SHE)	mV	-	60-390	34	127	35	21	5	52**	18	185	105	8	125	12	18	222	253	62	122	164
SO_4^{2-}	mg/L	0.25	1.2-97	28	0.33	<.25	<.25	<.25	<.25	<.25	<.25	0.26	<.25	4.3	<.25	<.25	4.3	6.2	1.7	<.25	<.25
Cl^-	mg/L	0.05	1.8-24	5.6	18	4.9	20	32	16	9.8	31	12	13	17	18	26	4.4	4.7	10	28	20
DN	mg/L	0.5	1.7-14	0.5	20	61	9.7	5.5	43	22	9.1	0.5	14	0.7	17	14	<.5	<.5	0.5	11	11
DOC	mg/L	0.5	5.0-6.9	1.2	1.4	8.5	2.6	2.3	7.4	4.4	1.4	1.1	2.6	1.5	3.5	2.5	1.0	1.0	0.9	1.6	1.5
NH_4^+	mgN/L	0.01	1.3-15	0.61	23	63	10	5.5	44	25	9.3	0.63	16	0.49	19	15	0.05	0.09	0.60	12	12
PO_4^{3-}	mgP/L	0.005	0.005	0.92	0.03	1.8	0.65	0.77	1.4	0.76	0.01	0.32	0.52	0.02	0.52	0.53	0.01	0.01	0.26	0.03	0.02
As	µg/L	0.1	15-508	135	22	513	352	337	452	401	0.9	6.2	80	4.3	266	58	1.0	<.1	6.2	0.6	0.7
As(III)	µg/L	0.1	11-450	135	21	489	346	320	416	372	0.3	6.1	76	3.8	262	58	0.3	<.1	6.0	0.5	0.4
Fe	mg/L	0.05	<.05-14	12	0.54	14	12	20	14	13	<.05	16	8.9	0.44	10	9.9	0.07	0.07	24	0.75	0.08
Mn	mg/L	0.005	3.2-3.9	0.66	1.5	0.15	0.21	0.17	0.16	0.50	1.5	1.0	3.6	2.7	1.0	2.5	2.4	0.28	1.5	1.9	1.1
P_{tot}	mg/L	0.02	0.02	1.1	0.09	2.1	0.70	0.89	1.5	0.77	0.06	0.38	0.60	0.06	0.52	0.59	0.03	0.03	0.29	0.06	0.09
S_{tot}	mg/L	0.1	0.4-35	11	<.1	<.1	<.1	<.1	<.1	<.1	<.1	<.1	<.1	1.5	<.1	<.1	1.5	2.3	0.6	<.1	<.1
Si	mg/L	1	9-14	14	9	15	11	16	13	10	15	17	8	13	9	9	16	17	18	13	11
Sr	µg/L	1	531-537	356	302	478	470	399	397	490	584	231	489	599	475	522	252	198	270	213	360
Br	mg/L	0.04	0.16-0.20	<.04	0.16	0.19	0.08	0.10	0.11	0.18	0.09	0.13	0.10	0.09	0.09	0.11	0.16	0.14	0.19	0.16	0.09
Na	mg/L	0.5	5.3-13.4	5.7	19	11	15	13	13	10	14	42	10	17	9.4	9.8	32	34	25	9.4	9.9
K	mg/L	0.1	4.7-7.4	2.7	6.6	8.4	3	1.6	6.1	6.0	4.5	3.9	5.0	5.8	5.3	5.0	3.3	2.8	4.2	3.6	4.7
Ca	mg/L	0.1	151-181	121	24	92	124	123	64	96	110	30	98	67	100	101	31	21	31	123	108
Mg	mg/L	0.01	34-37	27	26	29	30	29	31	33	37	21	26	67	32	34	27	21	32	18	22
Ba	µg/L	0.2	269-403	520	469	540	640	352	438	76	342	236	146	110	108	137	102	63	173	48	70
C-alk	mmolHCO_3^-/L	0.1	8.0-12	8.7	5.8	14	11	9.6	11	12	9.7	5.8	9.2	9.8	10	9.3	5.4	4.3	6.3	8.3	8.1
CH_4	mg/L	<0.13	-	<.13	<.13	53	5	1.9	30	51	<.13	<.13	28	<.13	25	15	<.13	<.13	<.13	<.13	<.13

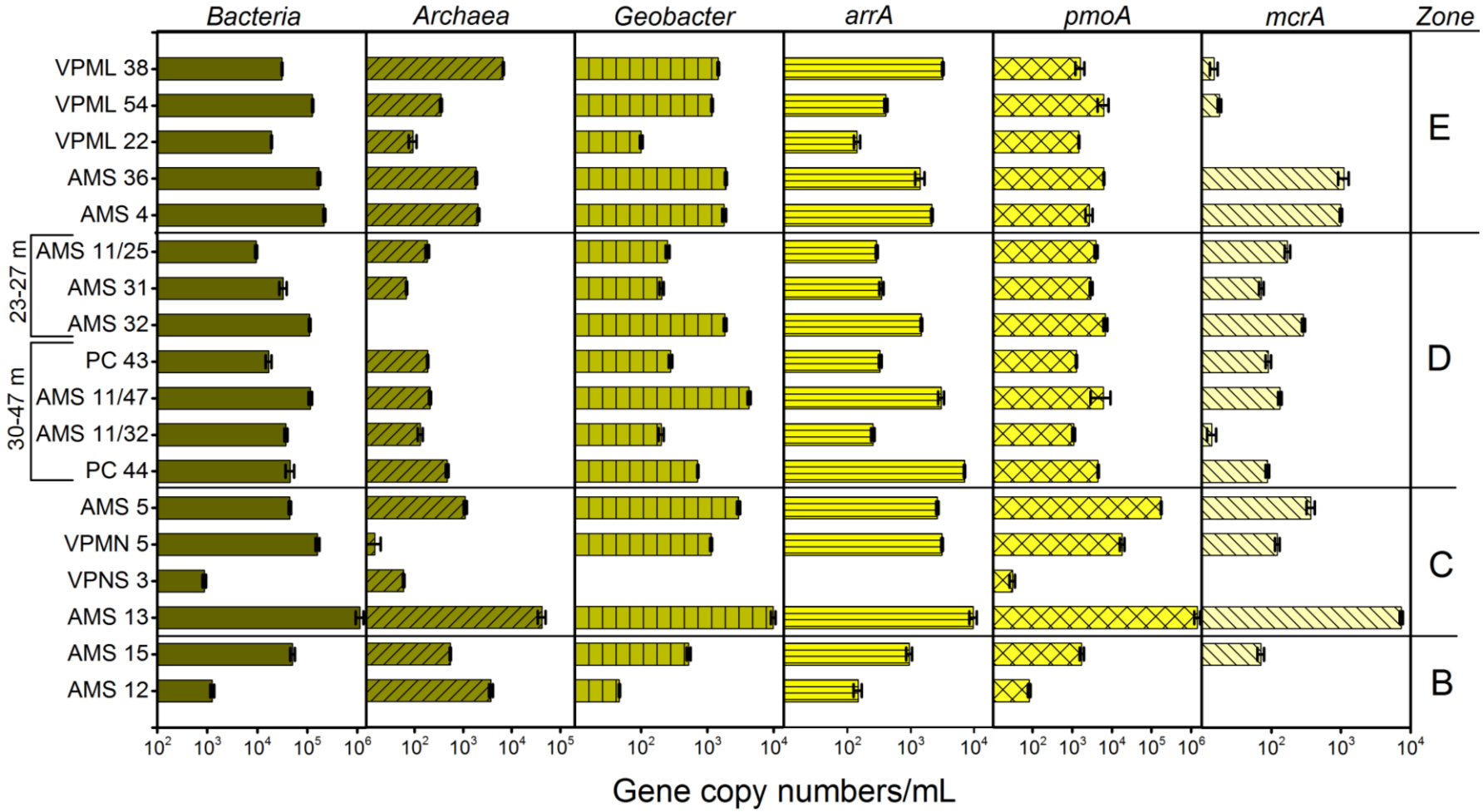
**Range from three riverbank samples collected in November 2018.*

***Parameters affected by sampling: the well was drying quickly and needed several cycles of pumping-refill*

Among the active microbial community (Figure 4.4), fermenters appeared to be the most abundant group of microorganisms, with the majority of taxa related to *Firmicutes*, *Chloroflexi*, and *Bacteroidetes* (Gupta et al., 2014; Kampmann et al., 2012). In addition, predicted fermentation dominated among all environmentally relevant metabolic pathways, as inferred from 16S rRNA amplicon sequences (Figure 4.5). A variety of organic acids and more bioavailable short-chain fatty acids, such as acetate, lactate, formate, or propionate, can be produced as a result of fermentation (Chapelle, 2000; McMahon & Chapelle, 1991). These fermentation products can fuel reductive dissolution and the release of As from Fe(III) (oxyhydr)oxides, enhancing reducing (low redox potential) conditions (Postma et al., 2007; Quicksall et al., 2008). Therefore, electron donors provided via fermentation can drive diverse heterotrophic microbial processes, including methanogenesis, SO_4^{2-} reduction, and, in particular, Fe(III) reduction.

The riverbank deposits are a source of SO_4^{2-}, impacting the biogeochemistry in zone B. The presence of SO_4^{2-} and saturation indices pointing toward FeS mineral precipitation (Stopelli et al., 2020) suggest that SO_4^{2-} reduction to sulfide (S^{2-}) occurs between the riverbank and the Holocene aquifer, causing S depletion from the solution. Taxa known to be involved in S-cycling, such as SO_4^{2-}-reducing bacteria which are affiliated with *Thermodesulfovibrionia* (Maki, 2015), were abundant, with 4% of the active microbial community in AMS12; these microorganisms may contribute to As immobilization in zone B, where up to 28 mg/L of SO_4^{2-} was measured. Sulfide (S^{2-}) produced as a result of dissimilatory SO_4^{2-} reduction can co-precipitate with Fe^{2+} and form greigite, mackinawite, or pyrite, which have a high affinity for As sorption (Bostick & Fendorf, 2003; Huerta-Diaz et al., 1998; Keimowitz et al., 2007; Wolthers et al., 2005). Arsenic can also be precipitated with either S^{2-} to form arsenic sulfide minerals or with S^{2-} and Fe^{2+} to form iron arsenic sulfides, such as arsenopyrite (Bostick et al., 2004; Kirk et al., 2004; O'Day et al., 2004). Thus, microbially driven SO_4^{2-} reduction in this zone might be a sink for As in groundwater and diminish its concentration to a certain extent.

Figure 4.3 DNA-based quantitative PCR analysis of bacterial 16S rRNA genes, archaeal 16S rRNA genes, Geobacter specific 16S rRNA genes, arsenate reductase genes (arrA), particulate methane monooxygenase genes (pmoA), and methyl-coenzyme M reductase subunit alpha genes (mcrA) in the groundwater wells of different zones (B-E) of the Van Phuc aquifer. Error bars show standard deviation from three measurements.



Considering the increased concentrations of dissolved Fe and As, it is not unexpected that Fe(II)-/As(III)- oxidizers thrive in this zone. In both wells of zone B, *Aquabacterium* was abundant (6.6% in AMS12 and 12% in AMS15). Different strains belonging to the genus *Aquabacterium* were shown to be capable of Fe(II) oxidation (Kalmbach et al., 1999; Zhang et al., 2017). Moreover, in many previous studies focusing on As-contaminated aquifers, *Aquabacterium* was found abundantly (Li et al., 2013, 2014; Sutton et al., 2009), implying that this taxon plays an important role in these reducing aquifers and may contribute to As immobilization. Arsenic-tolerant *Acinetobacter* was also highly abundant in this zone (3% in AMS12 and 6.5% in AMS15).

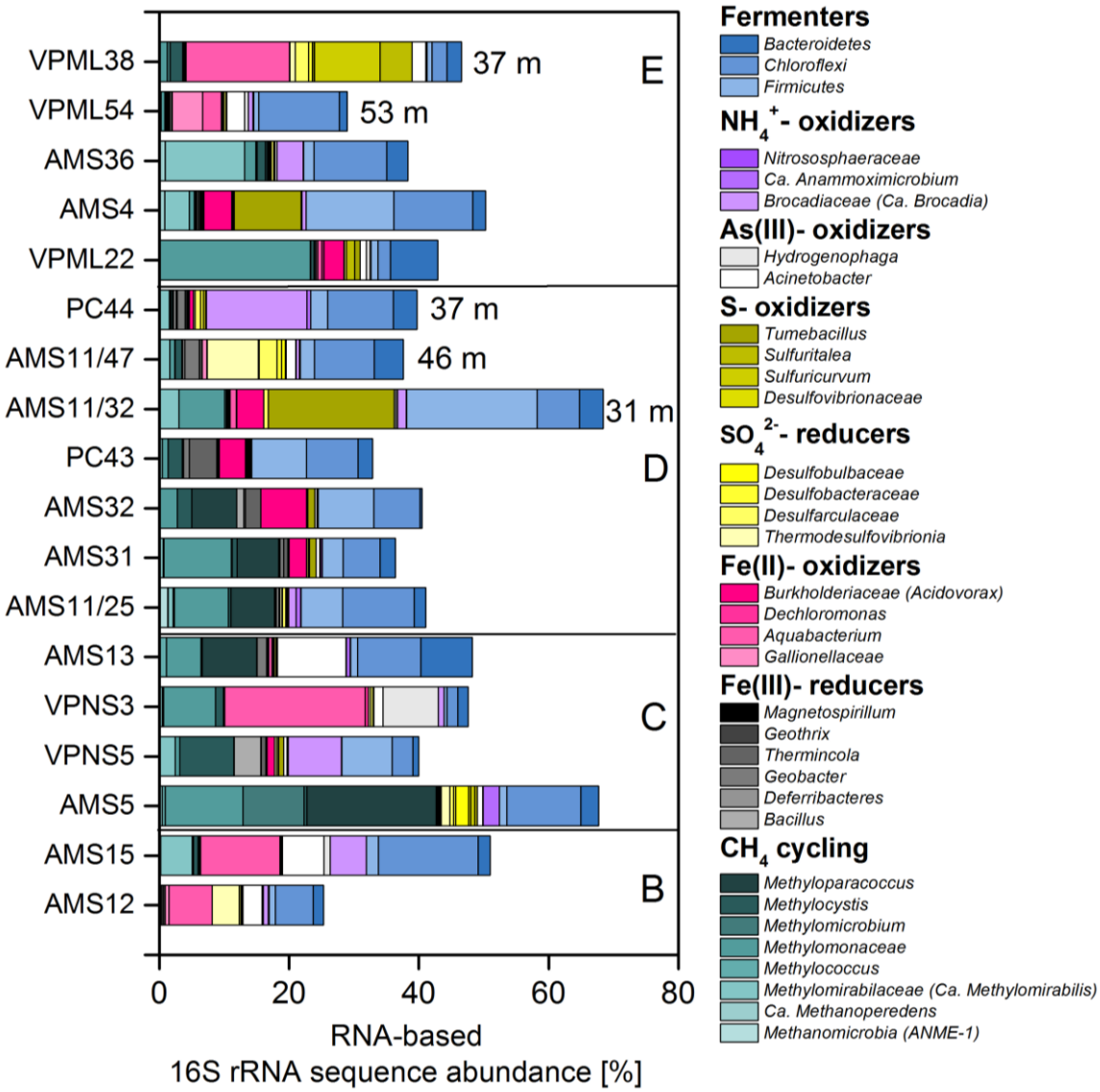


Figure 4.4 RNA-based 16S rRNA sequence abundance of selected taxa (including potential function) in groundwater samples of Van Phuc. Wells are divided into several zones according to Stopelli et al. (Stopelli et al., 2020): B: As-transport, C: As-mobilization, D: As-retardation, E: As-pristine/retardation. The wells had a depth between 20 and 27 m, except for those marked otherwise.

These microorganisms have been found to be very efficient in As removal from contaminated soil (Karn & Pan, 2016), and it is therefore possible that these bacteria also contributed to reducing As concentrations in groundwater sites from Van Phuc.

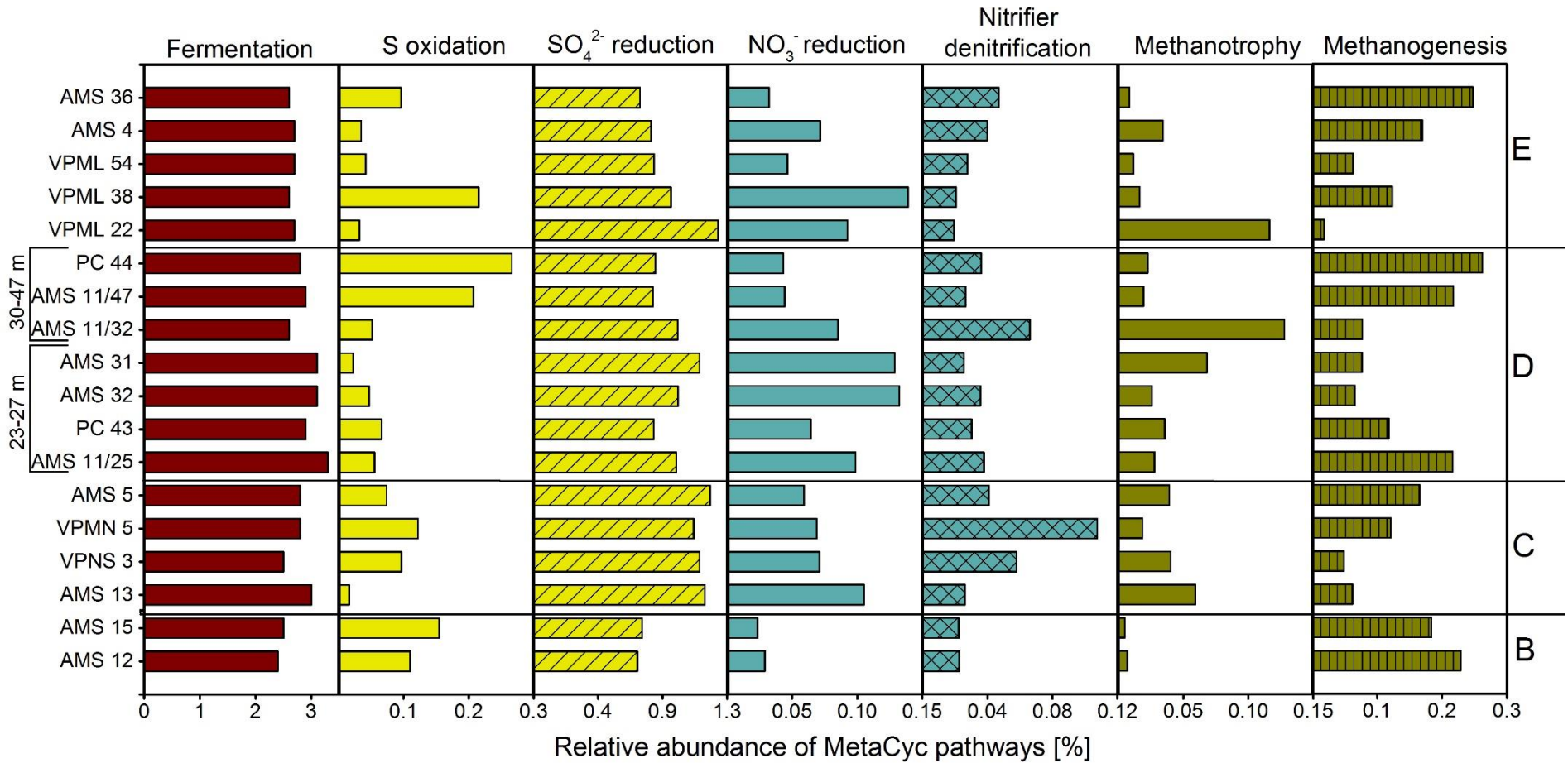
No CH_4 was detected in zone B and, in agreement with this finding, not many microorganisms related to methane cycling were present in the groundwater samples (Figure 4.4). Coherently, *mcr*A and *pmo*A genes related to methane cycling were also less abundant in these wells when compared to other zones (Figure 4.3).

To summarize zone B (Figure 4.7), carbon degradation and fermentation likely lead to As mobilization, while SO_4^{2-} reduction, Fe(II) and As(III) oxidation may promote As immobilization. Consequently, the co-occurrence of As mobilization and immobilization processes leads to a net transport of dissolved As from zone A to zone B.

4.4.2 Zone C: Methanogenic Holocene aquifer—further As mobilization

Four wells in the Holocene aquifer in zone C exhibited measurable CH_4 concentrations, ranging from 2 mg/L up to 53 mg/L. These wells were also characterized by low redox potentials (E_h +5 to +52 mV, SHE) and the highest concentrations of both dissolved As (337-513 µg/L) and dissolved Fe (12-20 mg/L) (Table 4.1). Moreover, in this zone, increased DOC values were present (2.3-8.5 mg/L) along with NH_4^+ (5.5-63 mg/L). Our previous study showed that the vertical infiltration of aquitard pore water is likely taking place in this zone (Stopelli et al., 2020). Aquitard sediments contain organic matter intercalations, in which pore water can be enriched in DOC and percolate into the aquifer along permeable intercalations (Eiche et al., 2008, 2017). The presence of additional C can stimulate microbially mediated Fe(III) mineral reduction and dissolution, thereby contributing to As mobilization. In fact, the enrichment of putative Fe(III)-reducers, such as *Bacillus* (up to 4%) and *Geobacter* (1.5%), was observed in some of the wells in zone C (Figure 4.4). In addition, relatively high *Geobacter* (up to 9.8 $x10^3$ $\pm 7.7 \times 10^2$/mL) and *arrA* (up to $9.7 \times 10^3 \pm 1.3 \times 10^3$/mL) gene copy numbers were found in most of the wells in zone C (Figure 4.3, Table S4.3). During Fe(III) reduction, OM-Fe-As complexes within aquifer sediments may also break up, promoting further C availability and As enrichment. Supplementary C in this zone likely supports fermentative processes, which is reflected in the high number of sequences affiliated with putative fermenters, with *Firmicutes* and *Bacteroidetes* reaching 8%, and *Chloroflexi* reaching 10% (Figure 4.4). Carbon degradation via fermentation probably contributes to the strong reducing conditions as well as the net As mobilization in this zone. Furthermore, fermentation processes provide substrates to fuel methanogenesis, leading to high dissolved CH_4 concentrations (up to 53 mg/L) (Table 4.1).

Figure 4.5 Relative abundance of predicted environmentally relevant microbial metabolic pathways in groundwater wells divided by zone (B-E). Metabolic potential was inferred from RNA-based 16S rRNA amplicon sequencing data and is based on MetaCyc pathways.



The presence of high concentrations of CH_4 in zone C is in agreement with a high abundance of diverse microbial taxa related to the CH_4 cycle, among which *Methyloparacoccus* (20%), *Methylomonaceae* (13%), *Methylomicrobium* (9.5%), and *Methylomirabilaceae* (2.5%) were found to be dominating (Figure 4.4). These results are further supported by high *pmoA* and *mcrA* gene copy numbers in this zone (Figure 4.3).

Within the highly reducing conditions of zone C, putative As(III)-oxidizers, such as *Acinetobacter* (Karn & Pan, 2016) and *Hydrogenophaga* (van den Hoven & Santini, 2004), were also abundant (up to 10% (Figure 4.4). These microorganisms can cope with high concentrations of As due to detoxification mechanisms, where As(III) is oxidized by a periplasmatic enzyme called arsenite oxidase (Chang et al., 2010). This self-defense mechanism leads to As(III) oxidation and can presumably decrease As mobility and promote its sorption into sediments. Furthermore, the potential Fe(II)-oxidizer *Aquabacterium* was highly abundant (> 20%) in well VPNS3, in which the dissolved Fe concentration was highest (20 mg/L). These microorganisms potentially immobilized part of the As that was released under highly reducing conditions while forming As-bearing Fe minerals.

To summarize zone C (Figure 4.7), the behavior of As is predominantly controlled by reductive processes that outcompete oxidative ones, since As concentrations in Holocene reach 300 to 500 µg/L. The relation between increased CH_4 and elevated Fe and As in the Holocene aquifer can be explained by the additional carbon input from aquitard pore water egression, fueling fermentation and methanogenic metabolisms and ultimately leading to an increased reduction of As-bearing Fe minerals.

4.4.3 Zone D: Redox transition from Holocene to Pleistocene aquifer—net As immobilization

The transition between the Holocene and Pleistocene aquifers is characterized by changing redox conditions, under which both processes of As mobilization as well as retention have been observed in zone D (Stopelli et al., 2020). In addition, many abiotic and biotic processes co-occur simultaneously, which makes this zone hydrochemically and microbially complex. Generally, shallow wells (23-27 m depth) were characterized by high dissolved As (58-401 µg/L), Fe (8.9-13 mg/L), NH_4^+ (15-25 mg/L), and CH_4 (15-51 mg/L) concentrations. Deeper wells (30-37 m depth), however, had no As, no CH_4, rather low NH_4^+ (0.49-9.3 mg/L), and almost no dissolved Fe. This is probably due to the fact that the egression of the aquitard pore water in zone C provides reducing conditions along the groundwater flow path mainly at 20-30 m, while at, deeper parts of the aquifer in zone D, “Pleistocene” -like, lesser reducing conditions prevail.

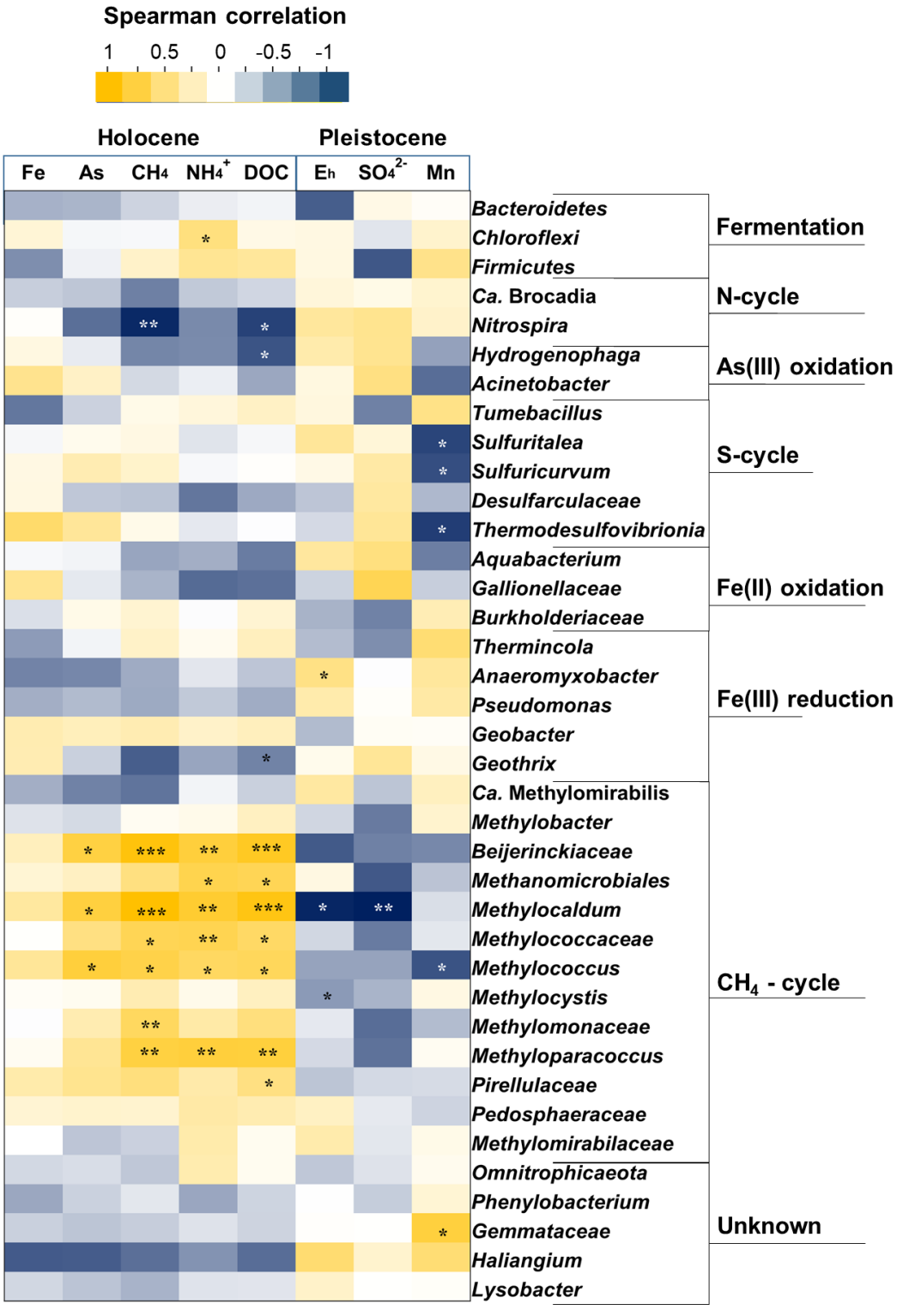


Figure 4.6 Heatmap of Spearman rank correlations of microbial taxa with hydrogeochemical parameters, such as dissolved Fe, As, CH_4, NH_4^+ and DOC (elevated concentrations more characteristic of Holocene aquifers) and E_h, SO_4^{2-} and Mn (elevated concentrations more characteristic of Pleistocene aquifers), with the most abundant taxa clustered by their putative functions. Significance levels (Benjamini-Hochberg corrected): $p < 0.1$ (*), $p < 0.05$ (**), $p < 0.001$(***)

The shallow wells AMS31, AMS32, PC43, and AMS11/25 showed a similar microbial community composition, which also corresponded with similar hydrogeochemical conditions. At these depths, DOC advected with Holocene groundwater from zone C was more abundant and likely promoted fermentative and methanogenic processes, as indicated by the high concentrations of CH_4 and NH_4^+ that are usually related to C degradation. In agreement with the higher C content, the microbial community composition among all the shallow wells was generally dominated by fermenters and methanotrophs. All wells with high CH_4 concentrations showed an increased abundance of the microorganisms involved in methanotrophy, which affiliated with *Methylomonaceae* (10% in AMS31) and *Methyloparacoccus* (up to 7% in AMS32 and AMS 11/25; Figure 4.4). Although the relative abundance of methanotrophs was lower in zone D compared to zone C, higher rates of CH_4 oxidation was expected in the redox transition of zone D. This is due to the higher availability of potential electron acceptors at the interface of the Holocene and Pleistocene aquifers. Several studies have shown that CH_4 may serve as an electron donor for anaerobic methanotrophs that couple CH_4 oxidation with Fe(III) reduction (Aromokeye et al., 2020; Cai et al., 2018; Ettwig et al., 2016). In fact, our latest study (Glodowska et al., submitted) demonstrated that this process can lead to a significant release of As from Fe- and As-bearing Van Phuc sediments, a process mediated by archaea affiliating with *Candidatus* Methanoperedens. However, anaerobic CH_4 oxidation can also be coupled with SO_4^{2-} reduction, a process driven by the syntrophic consortia of methanotrophic archaea (ANME-1, ANME-2a,b,c, and ANME-3) and SO_4^{2-}-reducing bacteria (Boetius et al., 2000; Knittel & Boetius, 2009; Milucka et al., 2012; Orphan et al., 2001; Scheller et al., 2016), which can potentially contribute to As immobilization via precipitation. Finally, a recent study by Leu et al. (Leu et al., 2020) revealed that CH_4 can serve as an electron donor for members of *Methanoperedenaceae*, while Mn(IV) can be used as an electron acceptor. This newly discovered pathway might be of relevance for As cycling, because Mn(IV) oxides present in sediments are effective oxidants (Scott & Morgan, 1995) and they can retain As in sediments; however, once Mn(IV) gets reduced to dissolved Mn^{2+}, As can be released into the groundwater. Therefore, CH_4-rich groundwater flowing through Pleistocene sediments containing Fe(III) and Mn (III/IV) (oxyhydr)oxides as well as the presence of SO_4^{2-}, can create favorable conditions for anaerobic CH_4 oxidation in the redox transition zone. These hydrogeochemical conditions, together with abundant methanotrophs, strongly imply that complex biogeochemical interactions between CH_4 and As are likely taking place in zone D. Considering the higher abundance of Fe(III) compared to Mn (III/IV) and SO_4^{2-}, Fe(III) is likely preferentially used as an electron acceptor and, in consequence, contributes to As mobilization.

However, it is important to bear in mind that the reduction of Mn(IV) is thermodynamically more favorable than the reduction of Fe(III) (Beal et al., 2009).

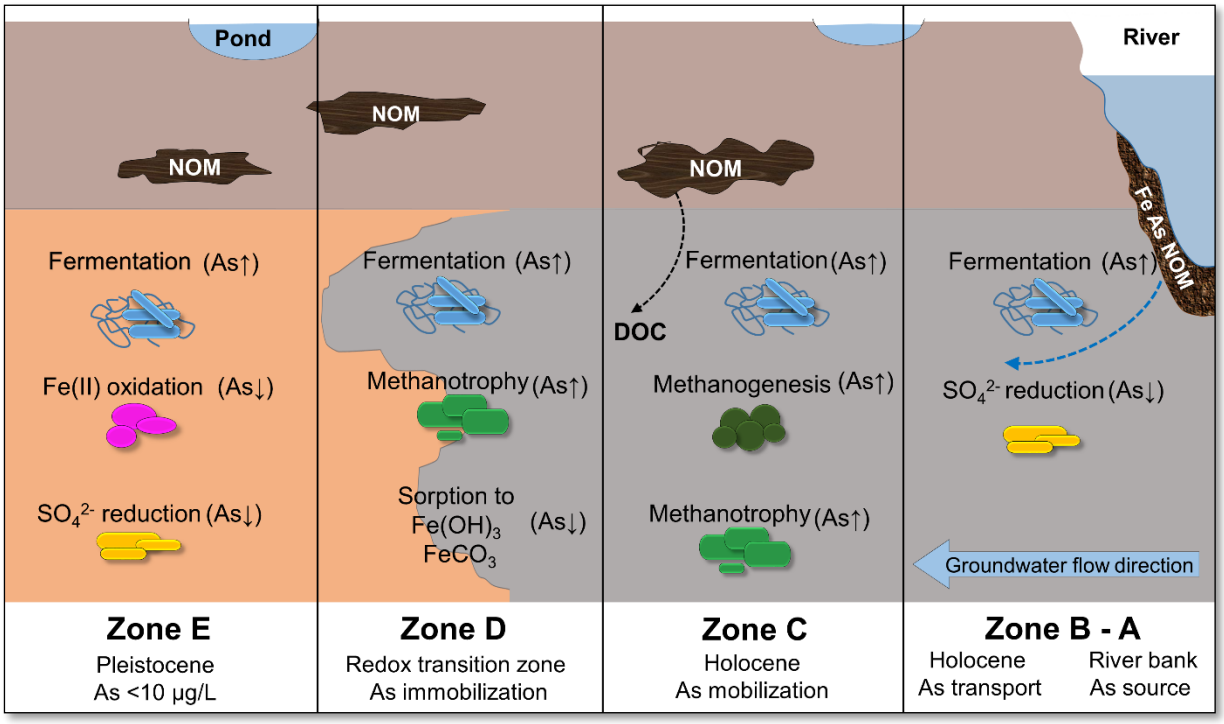


Figure 4.7 Two-dimensional cross-section of Van Phuc aquifers divided by hydrogeochemical zone (A-E), as reported in Stopelli et al., 2020. For each zone, the main microbial processes are indicated together with their net effect on As mobilization (As↑) or immobilization (As↓).

Furthermore, the wells being screened at different depths allowed for following the changes in their hydrochemistry and microbial community across a vertical profile and showed that entirely different processes seem to occur in deeper parts of zone D. In well AMS11, the bacterial population increased with depth by one order of magnitude: from $9.4 \times 10^3 \pm 4.5 \times 10^2$ 16S rRNA genes per mL at a depth of 25 m to $3.7 \times 10^4 \pm 2.7 \times 10^3$ at a depth of 32 m. At a depth of 25 m, only a very low relative abundance of microorganisms involved in S-cycling was identified, whereas, at a depth of 30 m, the groundwater was dominated by taxa related to *Tumebacillus* (19.5%). *Tumebacillus* has been previously shown to grow chemolithoautotrophically on inorganic sulfur compounds, such as sodium thiosulfate and sulfite, as sole electron donors (Steven et al., 2008). Furthermore, SO_4^{2-}-reducing bacteria, such as *Thermodesulfovibrionia* (8%) and *Desulfarculaceae* (3%), were abundant (Figure 4.4). In agreement, the predicted S-oxidation pathway appeared to be more abundant at deeper parts of zone D (Figure 4.5). The presence of microorganisms and predicted metabolic functions involved in S-cycling corroborates the hydrogeochemical data, as SO_4^{2-} was detected in the deepest wells (0.26 and 4.2 mg/L, respectively) (Table 4.1).

The composition of the *in situ* active microbial community (Figure 4.4) implies that oxidative processes are also occurring in zone D. An increased abundance (up to 7%) of putative NO_3^--dependent Fe^{2+}-oxidizers affiliating with *Acidovorax* was observed. These microorganisms were previously found in As-contaminated aquifers (Straub et al., 2004; Sutton et al., 2009), and they likely contribute to As removal through sorption or incorporation into freshly formed Fe(III) minerals, as was shown in a laboratory study (Hohmann et al., 2011). Furthermore, in some of the wells, taxa that affiliate with putative NH_4^+-oxidizers *Brocadiaceae* (mainly *Candidatus* Brocadia), (Jetten et al., 2015) were found abundantly, although NH_4^+ was present in all wells (concentrations ranging from 0.49 to 25 mg/L). In fact, *Brocadiaceae* accounted for up to 15% of the microbial community in well PC44, in which NH_4^+ concentrations were very low (0.49 mg/L), implying that decreased NH_4^+ concentrations were influenced by the activity of these microorganisms.

Generally, a retardation effect on As advection was observed in zone D (redox transition zone, Figure 4.7). However, the retardation capacity seems to depend on several microbial processes that can either contribute to the release of As from sediments, such as fermentation and methanotrophy, or retain it in sediments, such as Fe(II) oxidation and SO_4^{2-} reduction. Besides biological processes, abiotic processes can also largely influence As concentrations in this zone. High dissolved Mn concentrations were measured in the groundwater samples (Table 4.1), indicating that abiotic Fe(II) oxidation by Mn(IV) reduction can contribute to As immobilization in zone D. Therefore, the significant adsorption and incorporation of dissolved As into Fe(III) minerals—both newly formed and already present in Pleistocene sediments—are decreasing As groundwater concentrations (Stopelli et al., 2020). Furthermore, as an effect of methanotrophy, Fe^{2+} and CO_2 can be produced, which, in addition to Fe^{2+} and HCO_3^- flowing from the Holocene aquifer, could lead to the precipitation of Fe(II) carbonate and subsequent As sorption (Eiche et al., 2008; Sø et al., 2018).

4.4.4 Zone E: Pleistocene pristine aquifer—As immobilization

Dissolved As concentrations generally remained below the WHO guideline value of 10 µg/L in the Pleistocene aquifer (zone E). The wells in this zone were hydrogeochemically similar, presenting higher redox potentials from +122 to +253 mV (SHE), low dissolved As (< 0.1-1µg/L), and Fe (0.07-0.75 mg/L) as well as dissolved CH_4 below the detection limit (Table 4.1).

The *in situ* active microbial community in zone E was abundant in putative S-oxidizing bacteria mainly associated with *Sulfuricurvum* (10%), *Sulfuritalea* (5%), and *Tumebacillus* (10%). These bacteria were particularly abundant in well VPML38, in which elevated SO_4^{2-}

concentration (6.2 mg/L) were found among samples in zone E. Moreover, the highest relative abundance of a predicted S oxidation pathway was inferred from 16S rRNA amplicon sequences in this zone (Figure 4.5). These findings suggest that active S-cycling is taking place in zone E and, similarly to the deeper part of zone D, likely contributes to As sorption to FeS minerals and, thus, probably promotes As removal from groundwater.

Although no CH_4 was detected in these wells, AMS36 was dominated by a methanotrophic taxon related to *Methylomirabilaceae* (12%), while VPML22 was dominated by *Methylomonaceae* (23%). The high abundance of the *pmoA* gene further suggests that CH_4 oxidation might take place in this zone and contributes to a complete consumption of CH_4 transported from the Holocene aquifer, ultimately representing a cryptic CH_4 cycle similar to other cryptic cycles that have been demonstrated previously (Kappler & Bryce, 2017).

Finally, in well VPML38, potential Fe(II)-oxidizing bacteria, particularly those affiliating with *Aquabacterium*, represented more than 15% of the active microbial community. In addition, in the groundwater samples of other wells, increased abundances of Fe(II)-oxidizers related to *Gallionellaceae* (4.6%) and *Acidovorax* (4.3%) were observed (Figure 4.4). These microorganisms presumably contribute to As immobilization through the precipitation of Fe(III) minerals and a co-precipitation of As. To summarize, zone E is generally dominated by oxidative processes, efficiently maintaining low As concentrations in groundwater.

It is important to note that wells AMS11/47 in zone D and well VPML54 in zone E were screened at further depths (46 m and 53 m, respectively) compared to other wells. These wells reach a Pleistocene gravel layer underlying the sandy aquifers (Eiche et al., 2008; van Geen et al., 2013) and are characterized by high dissolved Fe (16 and 24 mg/L) but low As concentrations (6.2 µg/L; Table 4.1). At these depths, generally, processes that can lead to As immobilization seem to prevail. In AMS11/47, SO_4^{2-} reduction was indicated by a high abundance of *Thermodesulfovibrionia* (8%) and *Desulfarculaceae* (3%) (Figure 4.4) as well as a trace concentration of SO_4^{2-} (0.26 mg/L) (Table 4.1), while putative Fe(II)-oxidizers, such as *Gallionellaceae* (5%) and *Aquabacterium* (3%), and As(III)-oxidizers, such as *Acinetobacter* (3%), were dominating in VPML54.

4.4.5 Fermentation, CH_4 cycling, microbially mediated Fe(III), and SO_4^{2-} reduction dominate aquifer biogeochemistry

Diverse active microbial taxa and predicted metabolic pathways were identified in groundwater samples across Van Phuc aquifers. Fermenting microorganisms were the most abundant and omnipresent group. The correlation of active taxa with hydrogeochemical parameters (Figure

4.6) indicated that fermenting microorganisms are ubiquitous and can adapt both to Holocene and Pleistocene aquifer conditions. Therefore, fermentation seems to be a key biogeochemical process across the aquifers of Van Phuc. Pyruvate fermentation was the most common predicted fermentation pathway followed by homolactic, mixed acid, and heterolactic fermentation (Figure S4.1). As a result, a wide range of short-chain fatty and organic acids are likely produced, including acetate, lactate, formate, or propionate (Chapelle, 2000; McMahon & Chapelle, 1991). Thus, at our field sites, fermentation may provide easily bioavailable C compounds that further fuel diverse heterotrophic microbial processes, including reductive dissolution and As release from Fe(III) (oxyhydr)oxides (Postma et al., 2007; Quicksall et al., 2008).

Despite its high abundance in many As-contaminated aquifers worldwide, CH_4 remains largely unexplored when discussing its potential role in As (im)mobilization. Positive correlations between dissolved CH_4, Fe, and, by consequence, dissolved As have been previously reported (Dowling et al., 2002; Li et al., 2013; Liu et al., 2009; Postma et al., 2007), suggesting that methanogenesis can indirectly promote Fe(III) mineral reduction and As mobilization by providing CH_4 as an electron donor. Methanogenesis is directly fueled by fermentation products, and, in fact, all substrates necessary for CH_4 production, such as acetate, CO_2, and H_2, can be produced during fermentation (Alibardi & Cossu, 2016). Surprisingly, very high concentrations of CH_4 in some of the analyzed wells (up to 53 mg/L) did not correspond to the high relative abundances of methanogens nor *mcr*A genes. In fact, known methanogens accounted for as little as < 1% in all wells. A recent study showed that the methanotrophic population is mainly found in sediments rather than in water (Kuloyo et al., 2020), which is likely also true for methanogens. For this reason, we decided to explore the presence of methanogenic microorganisms in sediment samples, where they accounted for as much as 80% of the microbial community (Glodowska et al., unpublished). Despite the low abundance of methanogenic archaea in groundwater, the analysis of the main predicted metabolic pathways suggested that two types of methanogenesis take place in the aquifers: first, acetoclastic methanogenesis, where the main precursor is acetate:

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (1)$$

and second, hydrogenotrophic methanogenesis:

$$CO_2+4H_2 \rightarrow 2H_2O+CH_4 \quad (2)$$

where CO_2 is reduced to CH_4 (Goevert & Conrad, 2009). Metabolic pathways inferred from 16S rRNA amplicon sequences (Figure 4.5) suggested that the predicted acetoclastic methanogenesis is dominating in Van Phuc aquifers (Figure S4.2).

Methane cycling linked to As mobilization

Methane can be used as electron donor to fuel a wide range of microbially mediated processes, such as reductions of SO_4^{2-} (Boetius et al., 2000; Knittel & Boetius, 2009; Milucka et al., 2012; Orphan et al., 2001; Scheller et al., 2016), NO_3^- (Haroon et al., 2013), NO_2^- (Ettwig et al., 2010), and Mn(IV) (Leu et al., 2020). Most importantly, for our field site, anaerobic CH_4 oxidation can also be coupled with Fe(III) reduction (Aromokeye et al., 2020; Cai et al., 2018; Ettwig et al., 2016), a process that we confirmed within Van Phuc sediments and that can lead to significant As mobilization (Glodowska et al., submitted). Furthermore, microorganisms with the metabolic potential for CH_4 oxidation were abundantly present in most of the sampled wells (Figure 4.3 and 4.4). This might explain the large variability in the CH_4 concentrations measured in the groundwater samples in Van Phuc, ranging from < 0.13 mg/L to 53 mg/L. Methanotrophic communities at our field site are related to C degradation, as we found a strong correlation between methanotrophic bacteria and C degradation products, such as CH_4, DOC, and NH_4^+ (Figure 4.6). Some of these taxa are also significantly positively correlated with As, e.g. *Methylococcus* (r = 0.65), *Methylocaldum* (r = 0.64) or *Beijerinckiaceae* (r = 0.63) (Figure 4.6), which suggests their direct involvement in As mobilization. Thus, the correlation analysis confirmed our observation that microorganisms mediating CH_4 cycling were mainly active under conditions typical of Holocene aquifers and redox transition zones and less active in Pleistocene Mn-reducing aquifers, where they were negatively correlated with E_h, SO_4^{2-}, and dissolved Mn (Figure 4.6).

Low abundance of known Fe(III)-reducers

Active microbial taxa known to be involved in dissimilatory Fe(III) reduction, such as *Bacillus*, *Deferribacteres*, *Geobacter*, *Thermincola*, *Geothrix*, and *Magnetospirillum* were ubiquitous across the whole field site, although at rather low relative abundances (Figure 4.4). Many previous studies have shown the importance of Fe(III)-reducing bacteria in As mobilization (Héry et al., 2008; F. S. Islam, Boothman, et al., 2005; F. S. Islam, Pederick, et al., 2005; Kim et al., 2012; Ohtsuka et al., 2013). Nonetheless, in most of these studies, the *in situ* abundance of known Fe(III)-reducers was generally quite low (Héry et al., 2008; Kim et al., 2012; Li et al., 2013). Van Phuc's groundwater samples also showed a lower abundance of known Fe(III)-reducers, particularly when compared to dominant fermenters and CH_4 cycling microorganisms. This data suggests that either many unknown microorganisms in the community are capable of reducing Fe(III) or various microorganisms, such as methanotrophs, that have not been previously considered can actually contribute to Fe(III) reduction and As mobilization to a larger extent than known Fe(III)-reducers. Our previous study, where natural

organic matter was used as an electron donor, showed that diverse microorganisms contributed to Fe(III) reduction (Glodowska et al., 2020). However, when easily bioavailable C was added (acetate and lactate), mainly *Geobacter* was responsible for the reductive dissolution of sedimentary Fe(III) minerals. This strongly implies that a much larger microbial community than is currently known is involved in the reduction of Fe(III) minerals under natural conditions.

Sulfate reduction as a sink for As

The predicted genetic potential for SO_4^{2-} reduction and S oxidation appeared to be equally present in all wells (Figure 4.5). However, RNA-based 16S rRNA amplicon sequencing data showed that taxa identified as potential SO_4^{2-} reducers or S oxidizers were particularly abundant only in some of the wells (Figure 4.4), mainly in those where dissolved S species (dominated by SO_4^{2-}) were detected. Our hydrogeochemical data showed that the majority of wells for which SO_4^{2-} was reported were also characterized by low As concentrations, supporting our hypotheses that microbially mediated S cycling maintains low As concentrations in groundwater samples and that microbial SO_4^{2-} reduction (leading to sulfide production) is generally a sink for As in Van Phuc. This process seems to be particularly relevant at the redox transition zone and in the Pleistocene aquifer, where taxa involved in S-cycling appeared to be negatively correlated with dissolved Mn (Figure 4.6).

Cryptic N-cycle

Interestingly, pathways for predicted nitrate (NO_3^-) reduction and nitrifier denitrification were also inferred in all wells (Figure 4.5). At the same time, active microorganisms involved in the N-cycle were identified as NH_4^+-oxidizers in some of the wells (Figure 4.4), which are mostly associated with Pleistocene-like moderate reducing conditions (Figure 4.6). Nitrifier denitrification is a pathway in which NH_4^+ is oxidized to NO_2^-, which is subsequently reduced via NO and N_2O to N_2 (Wrage et al., 2001). This metabolic pathway is found in some autotrophic NH_4^+-oxidizers as well as in many CH_4-oxidizing bacteria (Stein & Klotz, 2011); therefore, its presence is likely related to methanotrophs, which were abundant in the microbial community. In fact, while our hydrogeochemical analyses did not show the presence of any measurable N species other than NH_4^+, our microbiological analyses indicated active N cycling. Such cycling is likely happening rapidly, therefore hampering the hydrogeochemical measurement of N species on top of NH_4^+, and could be responsible for the large variability in NH_4^+ concentrations in the groundwater samples. The implications of the N-cycle on As (im)mobilization, for instance, via nitrifier denitrification and microbially mediated NH_4^+ oxidation coupled with Fe(III) reduction (Feammox), deserve further investigation. In fact, Xiu

et al. (Xiu et al., 2020) proposed Feammox to be one of the mechanisms involved in As release in the western Hetao Basin.

Unexplored role of Mn in As immobilization

Finally, dissolved Mn appeared to be one of the decisive hydrogeochemical parameters affecting the active microbial community in Van Phuc aquifers (Figure 4.2 and Figure 4.6), yet, we could not identify microorganisms known to be directly involved in the Mn cycle. However, taxa affiliating with *Gemmataceae* ($r = 0.65$, $p < 0.1$) and *Haliangium* ($r = 0.47$, $p < 0.3$) showed a weak positive correlation with Mn (Figure 4.6), suggesting their potential involvement in the Mn cycle or associated processes. Moreover, a pathway of CH_4 oxidation coupled with Mn(IV) reduction has been recently described (Leu et al., 2020). This process might be relevant for the As mobilization reactions at the redox transition zone, considering the abundance and diversity of methanotrophs, the high concentration of dissolved CH_4, and the presence of Mn(IV) oxides within the sediments in this zone.

4.5 Conclusions

Understanding the interactions between microbiota and their hydrogeochemical environment is key in unraveling the biogeochemical network affecting As (im)mobilization. Our study shows that fermentation and methanogenesis, which have been largely overlooked in this context, are among the most important microbiological processes, indirectly favoring As mobilization. In addition, fermentation provides bioavailable C that further fuels various microbial metabolisms. Following methanogenesis, CH_4 is most likely oxidized by the reduction of various electron acceptors, such as As-bearing Fe(III) minerals that are especially abundant in the Pleistocene sediments. Methane oxidation is a process that has not been linked to the As cycle previously; however, a rich community of active CH_4 oxidizers was present in all zones of the studied aquifers, where it may contribute to As mobilization when coupled with Fe(III) and Mn(IV) reduction or to As immobilization coupled with SO_4^{2-} reduction.

At the same time, a number of oxidative metabolic processes co-exist and act as potential sinks for dissolved As, such as Fe(II) and/or As(III) oxidation. Moreover, the S cycle appears to be closely interlinked with dissolved As behavior. The presence of active SO_4^{2-} and S metabolizers at the redox transition and in the Pleistocene aquifer is related with lower As concentrations in groundwater samples. The formation of FeS, FeAsS, and AsS minerals is a result of an active microbially mediated S cycle and is likely responsible for the sorption and incorporation of As into these minerals.

These As (im)mobilization processes do not occur separately. In reality, complex biogeochemical interactions co-exist simultaneously and ultimately influence the groundwater's dissolved As concentrations. This is particularly relevant for the biogeochemistry taking place at redox transition zones, where fermentation, methanotrophy, SO_4^{2-} reduction, S oxidation, and Fe(II) oxidation may occur together (Figure 4.7).

Therefore, only by linking microbial and hydrogeochemical processes might we be able to explain the fate of dissolved As concentrations in both contaminated and pristine aquifers and especially its retardation across redox transition zones between Holocene and Pleistocene aquifers.

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4.7 Supplementary Information

Table S4.1 List of primers, primer sequences and thermal programs used for quantification of bacterial and archaeal 16S rRNA genes, arsenate reductase genes (*arrA*), Geobacter specific 16S rRNA genes, particulate methane monooxygenase genes (*pmoA*) and methyl-coenzyme M reductase subunit alpha genes (*mcrA*).

Specificity	Primer	Primer sequence (5´→ 3´)	Thermal program	References
16S rRNA genes of *Bacteria*	341f 534r	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	98°C - 2´; (98°C - 5´´; 60°C - 12´´ 95°C - 1´; 60°C - 1´) x 40; 60 – 95°C - 10´´	Muyzer et al., 1993
16S rRNA genes of *Archaea*	Ar109f Ar915r	ACK GCT GAG TAA CAC GT GTG CTC CCC CGC CAA TTC CT	98°C - 3´; (98°C - 5´´; 52°C - 12´´ 72°C - 15´) x 40; 98°C - 1´; 52°C - 1´; 52 – 95°C - 10´´	Großkopf et al., 1998 Stahl, 1991
arrA	arrA-F arrA-R	GGYSTGGGGCWSCGAYCC GGMASCCASTYGTGGGMC TT	95°C - 2´; (95°C - 30´´; 62°C - 40´´) x 40; 95°C - 1´; 62°C - 1´; (62 – 95°C - 10´´) x 67	Song et al., 2009
16S rRNA genes of *Geobacter*	Geo494F Geo825R	AGGAAGCACCGGCTAACTCC TACCCGCRACACCTAGT	50°C - 2´; 95°C- 10´; (95°C - 15´´; 56°C - 60´´) x 30	Holmes et al., 2002 Anderson et al., 1998
pmoA	A189f A682r	GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASGC	96°C - 5´; (94°C - 1´; 56°C - 1´; 72°C - 1´) x 38; 72°C - 5´	A. J. Holmes et al., 1995
mcrA	ME1f ME1r	GCMATGCARATHGGWATG TC TCATKGCRTAGTTDGGRTA GT	95°C - 5´; (95°C - 50´´; 54°C - 50´´ 72°C - 50´) x 34; 72°C - 10´	Hales et al., 1996

Table S4.2 Observed amplicon sequencing variants (ASVs) and alpha diversity indices in water samples from wells divided by geochemical zones as proposed in Stopelli et al. 2020 (Stopelli et al., 2020). Pielou's index (a measure of community evenness), Faith's Phylogenetic Diversity (a qualitative measure of community richness that incorporates phylogenetic relationships between the features) and Shannon's diversity index (a quantitative measure of community richness).

Zone	Well	Observed ASVs	Pielou e	Faith pd	Shannon
	AMS12	2830	0.86	190	9.90
B	**AMS15**	1906	0.79	130	8.64
	AMS13	1860	0.77	109	8.35
	VPNS3	882	0.68	76	6.65
	VPMN5	738	0.64	54	6.08
C	**AMS5**	1595	0.71	113	7.58
	PC44	1905	0.80	124	8.69
	AMS11/32	1766	0.71	120	7.64
	AMS11/47	1897	0.86	122	9.32
	PC43	2134	0.80	136	8.90
	AMS32	950	0.78	73	7.67
	AMS31	1841	0.79	119	8.54
D	**AMS11/25**	2260	0.79	139	8.81
	VPML38	1744	0.71	108	7.67
	VPML54	1545	0.85	96	8.98
	AMS36	1646	0.83	122	8.89
	AMS4	2052	0.82	138	8.98
E	**VPML22**	1097	0.69	81	6.96

Table S4.3 Quantitative PCR analysis of bacterial and archaeal 16S rRNA genes, arsenate reductase genes (*arrA*), *Geobacter* specific 16S rRNA genes, particulate methane monooxygenase genes (*pmoA*) and methyl-coenzyme M reductase subunit alpha genes (*mcrA*). The data is shown as gene copy numbers/mL in the groundwater samples from different geochemical zones of the Van Phuc aquifers (Stopelli et al., 2020).

Zone	Well	*Bacteria*	*Archaea*	*arrA*	*Geobacter*	*mcrA*	*pmoA*
	AMS12	1256 ± 120	3727 ± 346	146 ± 21	47.1 ± 0.8	1 ± 0	82 ± 6
B	**AMS15**	50610 ± 5553	542 ± 16	949 ± 95	522.4 ± 34	71 ± 8	1736 ± 214
	AMS13	1132444 ± 197401	42118 ± 7799	9765 ± 1300	9808 ± 770	7390 ± 395	1444555 ± 248776
	VPN3	870 ± 74	59 ± 2	6 ± 1	6.3 ± 0.2	2 ± 1	31 ± 5
	VPMN5	159500 ± 14110	15 ± 5	3127 ± 83	1136 ± 36	122 ± 9	18243 ± 2288
C	**AMS5**	44786 ± 2398	1111 ± 84	2625 ±107	2955 ± 148	369 ± 48	174733 ± 5774
	PC44	45676 ± 9053	468 ± 29	7092 ± 168	713 ± 13	87 ± 5	4481 ± 144
	AMS 11/32	37296 ± 2750	131 ± 15	252 ± 15	201 ± 18	14 ± 2	1087 ± 91
	AMS 11/47	114633 ± 8528	207 ± 8	3051 ± 324	4271 ± 235	133 ± 7	6070 ± 3195
	PC43	16916 ± 2282	185 ± 4	328 ± 16	278 ± 17	91 ± 7	1267 ± 54
	AMS32	111100 ± 4258	8 ± 1	1477 ± 42	1839 ± 88	290 ± 15	6879 ± 782
	AMS31	32923 ± 5563	67 ± 2	346 ± 26	204 ± 14	72 ± 5	2991 ± 229
D	**AMS 11/25**	9467 ± 453	182 ± 16	290 ± 12	250 ± 16	171 ± 15	3954 ± 356
	VPML38	31283 ± 585	347 ± 14	404 ± 22	1180 ± 30	18 ± 1	6274 ± 1927
	VPML54	126733 ± 3190	6657 ± 174	3220 ± 102	1451 ± 41	15 ± 2	1613 ± 421
	AMS36	170866 ± 10651	1862 ± 74	1417 ± 240	1903 ± 64	1104 ± 191	6266 ± 172
	AMS4	217300 ± 9945	2044 ± 88	2154 ± 54	1793 ± 116	996 ± 36	2690 ± 522
E	**VPML22**	19283 ± 42	91 ± 17	142 ± 15	100 ± 4	3 ± 1	1466 ± 36

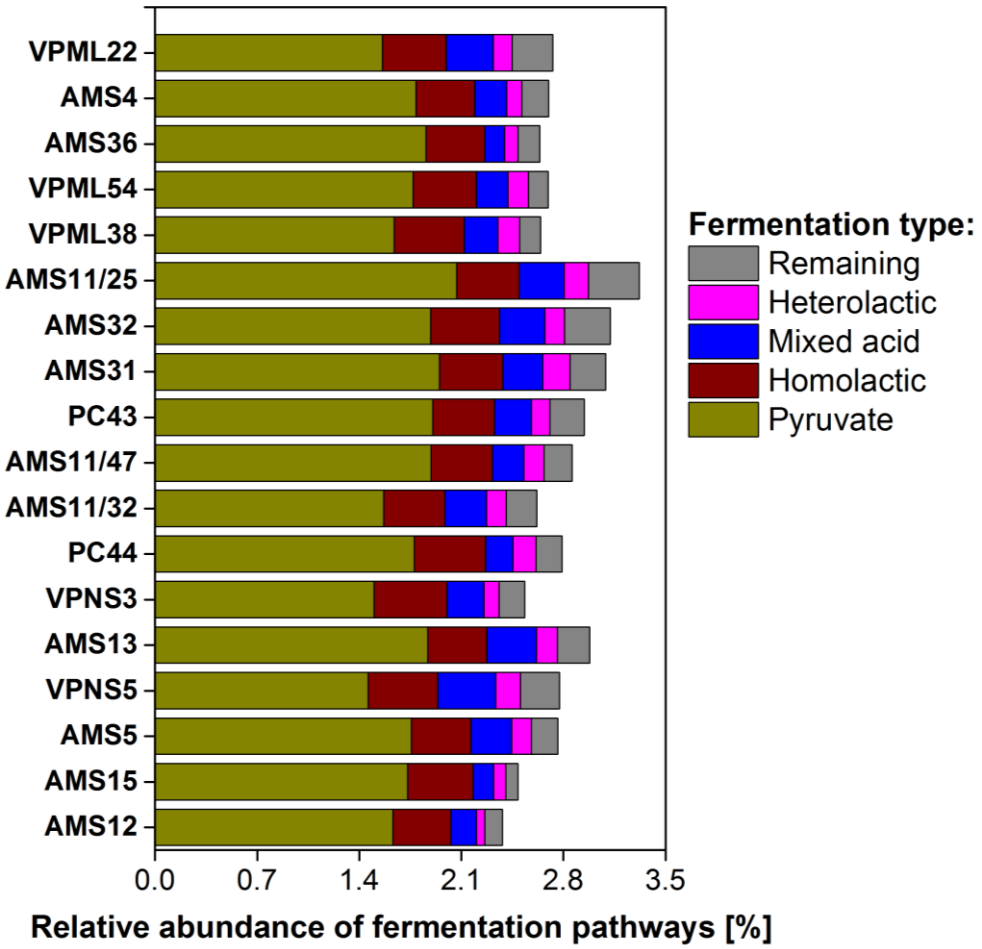

Figure S4.1 Relative abundance of predicted fermentation pathways in groundwater samples as inferred from 16S rRNA amplicon sequences based on MetaCyc pathways.

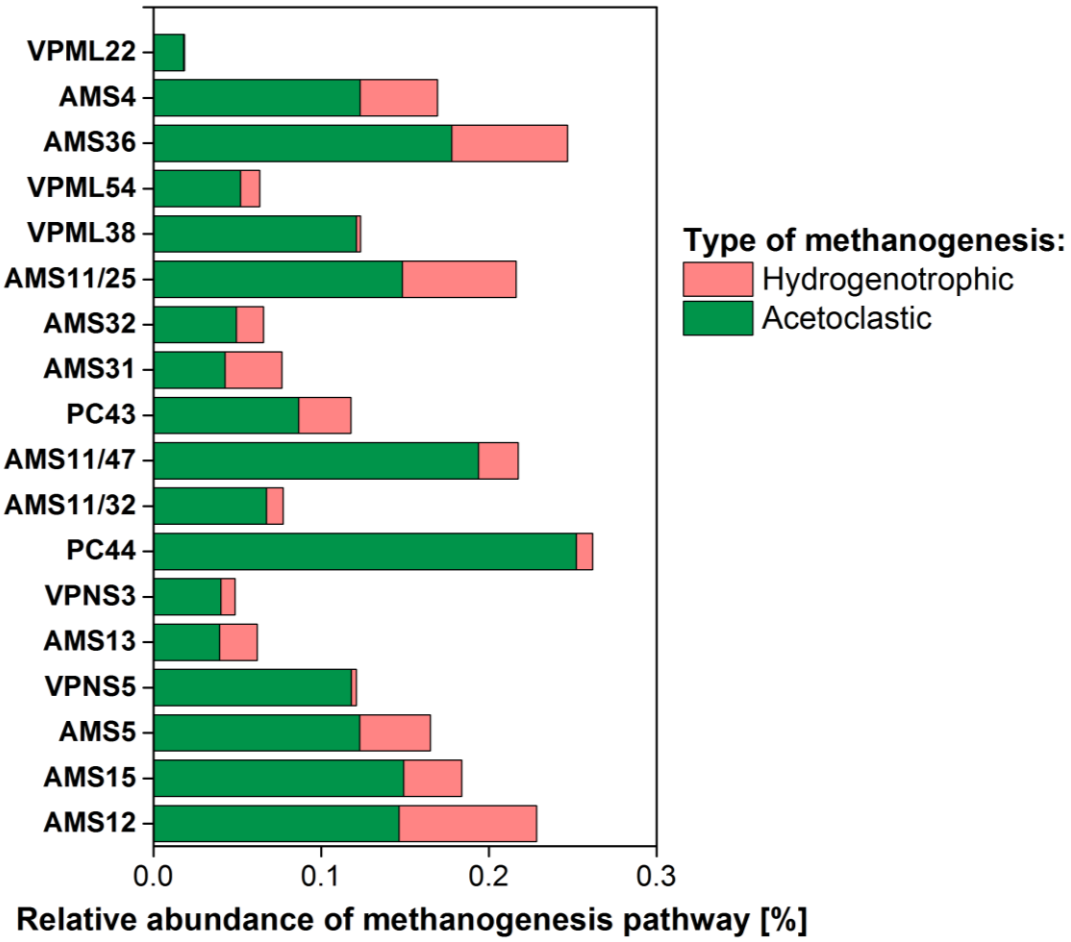

Figure S4.2 Relative abundance of predicted methanogenic pathways in groundwater samples as inferred from 16S rRNA amplicon sequences based on MetaCyc pathways.

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Chapter 5: Conclusions and Outlook

Arsenic biogeochemistry is strongly affected by various abiotic and biotic processes. Identifying and understanding these processes are the key to explaining the behavior of this potent carcinogen, (Naujokas et al., 2013) and to predict its fate in groundwater, which is often a source of drinking water. It is not enough, however, to investigate As groundwater chemistry or hydrology, as neither sediment mineralogy nor microbially mediated As transformations separately. Only by combining knowledge from different disciplines and understanding interactions between various processes may allow us to develop an integral and generally applicable understanding of As dynamics, particularly under complex redox conditions. This PhD thesis is a part of such an interdisciplinary effort, that overall broadened our understanding of As biogeochemical cycling and filled some knowledge gaps about microbial contribution to this cycle.

The main goal of this PhD thesis was to increase current knowledge about microbial processes that can affect As behavior in groundwater. To achieve this goal, the role of organic matter (OM) in microbially mediated Fe(III) reduction was investigated. It is particularly important to understand the specific contributions of Fe(III)-reducing microorganisms to As release, since Fe(III) reduction is a well-known pathway of As release to groundwater. The study presented in Chapter 2 showed that microbial Fe(III) reduction rate and extent strongly depend on type of carbon (C) source provided to the indigenous microbial Fe(III)-reducing community. In regards to the contribution of Fe(III) reducers in As mobilization, clearly it was shown to be significant in laboratory study. Yet, field data demonstrated rather low abundance of known Fe(III)-reducing microbes compared to other taxa in groundwater microbial communities (Chapter 5). This result implies that microbial Fe(III) reduction is mediated by more than a few known Fe(III)-reducing microbial species and can be driven by a diverse group of microorganisms.

Another objective of this work was to evaluate the role of methane (CH_4) as potential electron donor. High concentrations of (CH_4), as well as the abundance and diversity of methanotrophic and methanogenic microbial communities in groundwater and sediments from Van Phuc, strongly implied the importance of CH_4 cycling in these aquifers. These chemical and microbiological observations motivated the investigation of anaerobic CH_4 oxidation coupled to Fe(III) mineral reduction. This study, for the first time, highlighted a direct link between CH_4 and As cycling, and demonstrated that CH_4 can drive Fe(III) mineral reduction and As

mobilization. Furthermore, CH_4 can serve as an electron donor for a wide range of microbially mediated processes that can also affect As mobility.

Finally, the last objective of this PhD thesis was to translate the knowledge gathered from laboratory studies into environmental context. The aim of Chapter 4 was therefore to determine the dominate microbial processes in Van Phuc groundwater aquifers and the impact of these processes on As mobility. Examining active microbial communities indeed validated the importance of CH_4 cycling in As fate and underlined the role of microbial processes such as fermentation, methanogenesis and methanotrophy, which have not been previously considered in detail in relation to As cycling. Furthermore, combining microbial community analysis with hydrogeochemical data provided evidence of an active S-cycle, with sulfide mineral formation potentially as a sink for As in groundwater. Overall, the analysis of complex biogeochemical interactions demonstrated that simultaneous occurrence of various biological and abiotic processes ultimately controls groundwater As concentration.

5.1 Importance of carbon for microbially mediated Fe(III) reduction and As mobilization

Many mechanisms of As release to groundwater have been proposed (Farhana S. Islam et al., 2004); yet, the most commonly accepted mechanism of As mobilization is that As bearing Fe(III) (oxyhydr)oxide minerals are reduced by anaerobic Fe(III)-reducing microorganisms, a process that is coupled to oxidation of organic carbon (Chatain et al., 2005; F. S. Islam, Pederick, et al., 2005; Farhana S. Islam et al., 2004). Although high enrichment of Fe(III)-reducing microbes and efficient As mobilization was proven in many laboratory studies, the abundance of microorganisms known to mediate this process was found to be rather low in actual As-contaminated aquifers (Héry et al., 2008; Kim et al., 2012; P. Li et al., 2013). Also, the *in-situ* study presented in Chapter 4 showed that these microorganisms represented only small fraction of active microbial communities in groundwater. The abundance and activity of Fe(III)-reducing microbes are highly dependent on quantity and bioavailability of organic C (Chapter 2), required as a source of energy and electrons for metabolic activity (Mailloux et al., 2013; Rowland et al., 2007). The C availability in As-contaminated aquifers, however, might be limited to poorly bioavailable C compounds. In fact, in many aquifers across South and Southeast Asia, embedded organic rich layers consisting of peat lenses and poorly degraded plant residues were characterized as the main C sources (Anawar et al., 2003; Buschmann et al., 2007; McArthur et al., 2004). This type of organic C is considered rather resistant to

chemical and biological degradation (Marschner et al., 2008; Ruiz-Dueñas & Martínez, 2009), and therefore largely unvailable for majority of microorganisms. Only specialized C degrading microorganisms can break down complex organic biopolymers such as lignin and cellulose via enzymatic reactions under anaerobic conditions (J. Li et al., 2009; Pérez et al., 2002). Nonetheless, organic matter decay and further C degradation processes such as fermentation can provide easily bioavailable C compounds. A variety of organic acids and more bioavailable short-chain fatty acids such as acetate, lactate, formate or propionate, can be produced via fermentation (Chapelle, 2000; McMahon & Chapelle, 1991). In most of laboratory studies, high concentrations (5-50 mM) of these easily bioavailable carbon sources such as acetate, lactate, glucose, polypepton or urea were used to investigate potential of Fe(III)-reducing microbes to mobilize As (Duan et al., 2008; Gault et al., 2016; Neidhardt et al., 2014; Radloff et al., 2007; Rowland et al., 2007). These conditions indeed usually lead to the enrichment of Fe(III)-reducers, high rates of Fe(III) reduction and consequently, substantial As mobilization. Although the commonly used, easily bioavailable C-sources are useful as a proxy in simple laboratory experiments, these C sources do not represent environmental conditions and usually overestimated Fe(III) reduction and As mobilization rates, particularly when used in unrealistic concentrations. Knowing the type of available C in the environment helps to better estimate the potential as a fuel for microbial Fe(III) reduction. The study presented in Chapter 2 aimed to use C extracted directly from sediments as a more environmentally relevant C source, with supplied concentrations closer to those observed in the field. Results of this study confirmed the hypothesis that easily bioavailable acetate and lactate trigger Fe(III) reduction much faster and to a higher extent compared to natural organic matter (NOM). Moreover, it showed that bioavailable C is mainly used by a single taxon (*Geobacter* spp.). On the contrary, extracted NOM led to much slower Fe(III) reduction, sustained more diverse microbial communities for much longer, but ultimately mobilized higher concentrations of As compared to microcosms supplied with acetate and lactate Geobacter-dominated community (Figure 5.1).

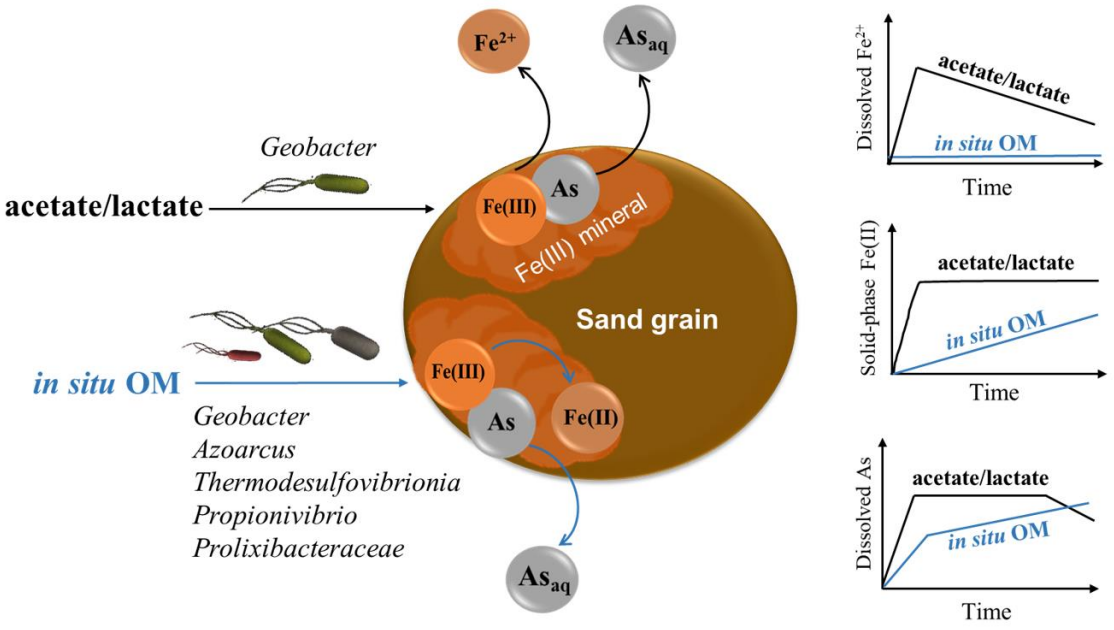

Figure 5.1 Overview of how natural organic matter (NOM) affect Fe(III) reducation, As mobilization and microbial community composition compared to bioavialiable C sources such as acetate and lactate.

Access to bioavailable C resources in aquifers might be limited and competition for it among heterotrophic microorganisms is likely very high. This may be a key reason why the abundance of known Fe(III)-reducers in groundwater is rather low. Based on our studies (Chapter 4) fermenters, methanogens and methanotrophs are dominating and ubiquitous microbial groups across Van Phuc aquifers. These microorganisms might be more adaptable and better equipped to use various (less available) C sources, efficiently suppressing more selective Fe(III)-reducers. Additionally, only several taxa such as *Geobacter* sp. (F. S. Islam, Pederick, et al., 2005), *Shewanella* (Cummings et al., 1999) or *Geothrix* sp. (F. S. Islam, Boothman, et al., 2005) have been shown to use Fe(III) as electron acceptors in As-contaminated environments. However, *in situ* microbial community present in groundwater aquifers under Fe(III) reducing conditions, consist of diverse microorganisms of unexplored metabolic potential, implying that many of these unknown taxa can in fact also reduce Fe(III). Furthermore, in recent years methanotrophic archaea has been shown to be capable of using Fe(III) as electron acceptors (Cai et al., 2018; Ettwig et al., 2016), further confirming that Fe(III)-reducers may in reality be much more abundant, but the identity of these species is still unknown.

5.2 Methane as a driver for microbially mediated As (im)mobilization

Methane was found in the majority of As-contaminated aquifers of South and Southeast Asia, often at flammable concentrations (Harvey et al., 2002; Jessen et al., 2008; Liu et al., 2009; Polizzotto et al., 2005; Postma et al., 2007, 2012). The presence of CH_4 is usually linked to microbial decomposition of C (Liu et al., 2009) and generally reducing conditions (Postma et al., 2007; Quicksall et al., 2008). Consequently, several previous studies showed a positive correlation between dissolved Fe and CH_4 and subsequently positive correlation between CH_4 and As (Dowling et al., 2002; P. Li et al., 2013; Liu et al., 2009; Postma et al., 2007). Only recently was it demonstrated that CH_4 can also serve as an electron donor for anaerobic methanotrophs able to couple CH_4 oxidation to Fe(III) reduction (Aromokeye et al., 2020; Cai et al., 2018; Ettwig et al., 2016), a process mediated by archaea related to *Candidatus* Methanoperedens. The presence of Fe(III) (oxyhydr)oxides, high concentrations of dissolved CH_4 and an abundance of methanogenic and methanotrophic microorganisms in As-rich aquifers strongly implies the direct role of CH_4 in Fe(III) reduction and As release (Kirk et al., 2004; P. Li et al., 2013, 2014; Paul et al., 2015). Thus, the study presented in Chapter 3 aimed to better understand this link and prove that CH_4 oxidation coupled to reductive dissolution of As-bearing Fe(III) (oxyhydr)oxide minerals can lead to As release. The results of this study provide evidence for a completely new mechanism of As mobilization that may be relevant for many aquifers across South and Southeast Asia. Although CH_4 can also be used as electron donor to fuel NO_3^- (Haroon et al., 2013) and NO_2^- (Ettwig et al., 2010) reduction, these processes are of little relevance for As mobility, particularly in Van Phuc groundwater where no NO_3^- or NO_2^- were measured. However, previously reported anaerobic oxidation of CH_4 coupled to sulfate (SO_4^{-2}) reduction, a process driven by syntrophic consortia of methanotrophic archaea (ANME-1; ANME-2a,b,c; and ANME-3) and sulfate-reducing bacteria (Boetius et al., 2000; Knittel & Boetius, 2009; Milucka et al., 2012; Orphan et al., 2001; Scheller et al., 2016), might influence the fate of As in groundwater in the system studies here. Until today there was no study that linked CH_4 driven SO_4^{-2} reduction with As immobilization; nevertheless, this process might be a valid sink of As in groundwater (Fig. 5.1). A number of studies demonstrated that sulfide minerals produced via microbially mediated SO_4^{-2} reduction, can immobilize As by forming FeS minerals, which have high affinity for As sorption (Bostick & Fendorf, 2003). Arsenic can also be directly incorporated into the structure of FeAsS or AsS minerals (Newman et al., 1997). Either way, SO_4^{-2} reduction is generally associated with decreased As concentration in water (Buschmann & Berg, 2009; Kirk et al., 2004; Rittle et al., 1995) and it has been proposed as remediation strategy for As contaminated groundwater (Keimowitz et al.,

2007). Methane driven SO_4^{-2} reduction is likely taking place in many As-contaminated aquifers, particularly in those where dissolved SO_4^{2-} and CH_4 occur simultaneously; however, at the moment this process remains completely unexplored and is in need of further investigation.

Another elemental cycle which can be linked to both CH_4 oxidation and As mobilization is the manganese (Mn) cycle. Methane oxidation coupled to Mn(IV) reduction (ΔG_0'= -790 kJ/mol) is thermodynamically much more favorable than SO_4^{2-} (ΔG_0'= -21 kJ/mol) and Fe(III) (ΔG_0'= -454 kJ/mol) reduction (in 't Zandt et al., 2018). Therefore, confirming that this process can be mediated by microorganisms was only a matter of time. In fact, several studies based on biogeochemical observations suggested that Mn(VI)-dependent anaerobic CH_4 oxidation takes place in marine sediments (Beal et al., 2009), as well as in freshwater and brackish wetland sediments (Segarra et al., 2013). However, only recently this process was confirmed in a freshwater sediment bioreactor, mediated by members of the family *Methanoperedenaceae* (Leu et al., 2020).

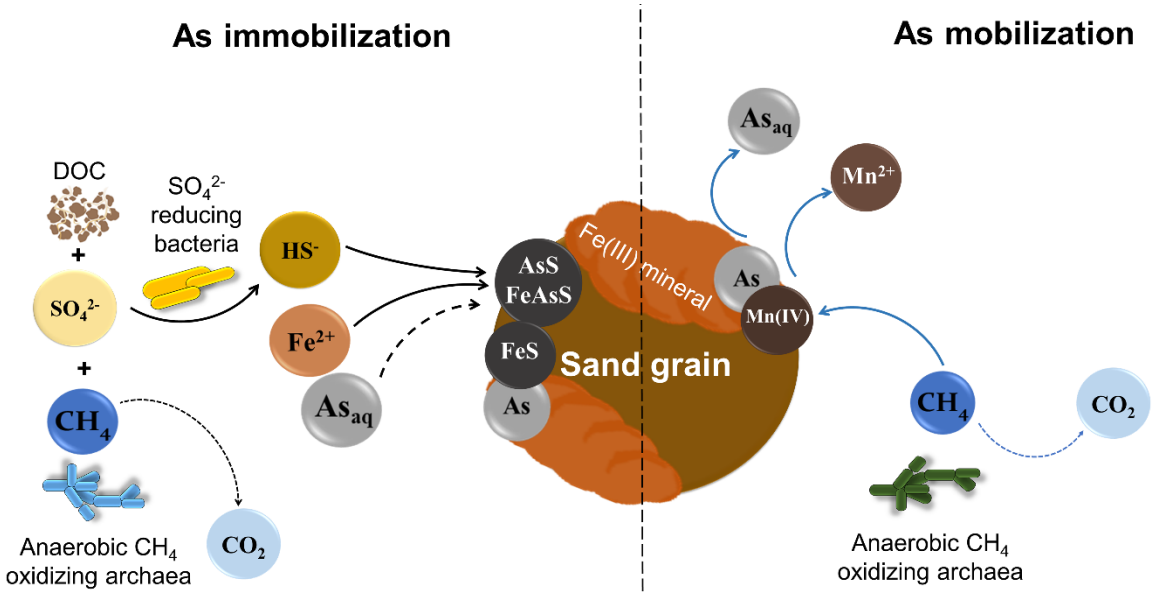

Figure 5.2 Potential mechanisms of As immobilization via anaerobic CH_4 oxidation coupled to SO_4^{-2} reduction (left) and As mobilization via anaerobic CH_4 oxidation coupled to Mn(IV) reduction.

This newly discovered pathway may be of relevance for As mobility in groundwater because Mn(IV) oxides present in sediments are effective oxidants (Scott & Morgan, 1995), retaining As in sediments. However, once Mn(IV) is reduced to dissolved Mn^{2+}, As is no longer retained in sediments and can be released to the groundwater (Fig. 5.1). Although, Fe(III) (oxyhydr)oxides are generally more abundant constituent of aquifer sediments than Mn(IV) (oxyhydr)oxides, reduction of Mn(IV) is thermodynamically more favorable than reduction of Fe(III) (Segarra et al., 2013), thus, Mn(IV) may be preferentially used as an electron acceptor.

This process, alike CH_4 driven SO_4^{-2} reduction in the regard of As mobility, has never been examined and therefore merits further investigation.

5.3 Microbial processes dominating in Van Phuc aquifers

Multitudes of laboratory studies were conducted in order to explain the impact of various microbial processes on As mobility. These studies provided important information regarding the role of Fe(III)-reducing (Cummings et al., 1999; DiChristina et al., 2002; Holmes et al., 2002; F. S. Islam, Boothman, et al., 2005) and As(V)-reducing bacteria (Duan et al., 2008; Héry et al., 2008; Liao et al., 2011) in As mobilization. Separately, researchers investigated antagonistic processes such as microbially mediated Fe(II) (Hallbeck & Pedersen, 1990; Hashimoto et al., 2007; Hohmann et al., 2010; Katsoyiannis & Zouboulis, 2006; Hohmann et al., 2011) and As(III) oxidation (Garcia-Dominguez et al., 2008; Ike et al., 2008; Karn & Pan, 2016) as a sink for As. A lot of focus was also placed on SO_4^{-2}- reducing bacteria, since sulfide production often correlates to a decrease in aqueous As concentrations (Bostick & Fendorf, 2003; Newman et al., 1997, Kirk et al., 2004; Rittle et al., 1995). However, studies based on environmental observations as well as laboratory sediment incubations show that these group of microorganisms often co-exist (Héry et al., 2008; Lear et al., 2007; P. Li et al., 2013; Xiu et al., 2020), and therefore, it is likely the coupled activity of various microorganism that ultimately drives groundwater As concentration, rather than each process separately. Yet, very few studies to date have investigated the combined effect of these simultaneous processes *in-situ*, further attempting to explain complex biogeochemical interactions affecting As mobility (Xiu et al., 2020). The lack of studies investigating complex coupled biogeochemical cycles is understandable, as these studies pose major challenges, stemming from limited knowledge about uncultivated microorganism, their metabolic potential and environmental functions. Chapter 5 of this PhD thesis aimed to explain behavior of As across different types of aquifers in Van Phuc by combining active microbial communities with hydrogeochemical data. This study brought some important insights about microbial processes that were largely overlooked in regard to As biotransformation and mobility, but appear to be dominant in these aquifers. Processes such as fermentation and methanogenesis are extremely important for As fate in groundwater (Figure 5.3). As a consequence of fermentation, a wide range of short-chain fatty and organic acids can be produced (Chapelle, 2000; McMahon & Chapelle, 1991). These easily bioavailable C compounds can fuel various microbial processes, particularly microbial Fe(III) reduction, subsequently contributing to As mobilization. Furthermore, methanogenesis provides CH_4, which can serve as an electron donor (Chapter 4) for anaerobic methanotrophs

and depending on the electron acceptor, can mobilize or immobilize As (discussed in subchapter 5.2). Zones where SO_4^{2-} was measured (redox transition zone and Pleistocene aquifer), are generally characterized by low As concentrations, in agreement with previous studies, which showed that SO_4^{2-} reduction leads to As removal (Kirk et al., 2004; Rittle et al., 1995). In these zones, a high abundance of SO_4^{2-}- reducing bacteria were also observed among active microbial communities, strongly implying that FeS, AsS and FeAsA might be produced and efficiently retained As in sediments (Bostick & Fendorf, 2003; Newman et al., 1997). Several oxidative microbial processes, such as Fe(II) and As(III) oxidation, appeared to contribute to decreasing As concentration in groundwater, as microorganisms known to mediate these processes were enriched across Van Phuc aquifers. The source of oxidants for these processes, however, remain elusive since Van Phuc aquifers are anoxic and NO_3^- and NO_2^- are below measurable concentrations.

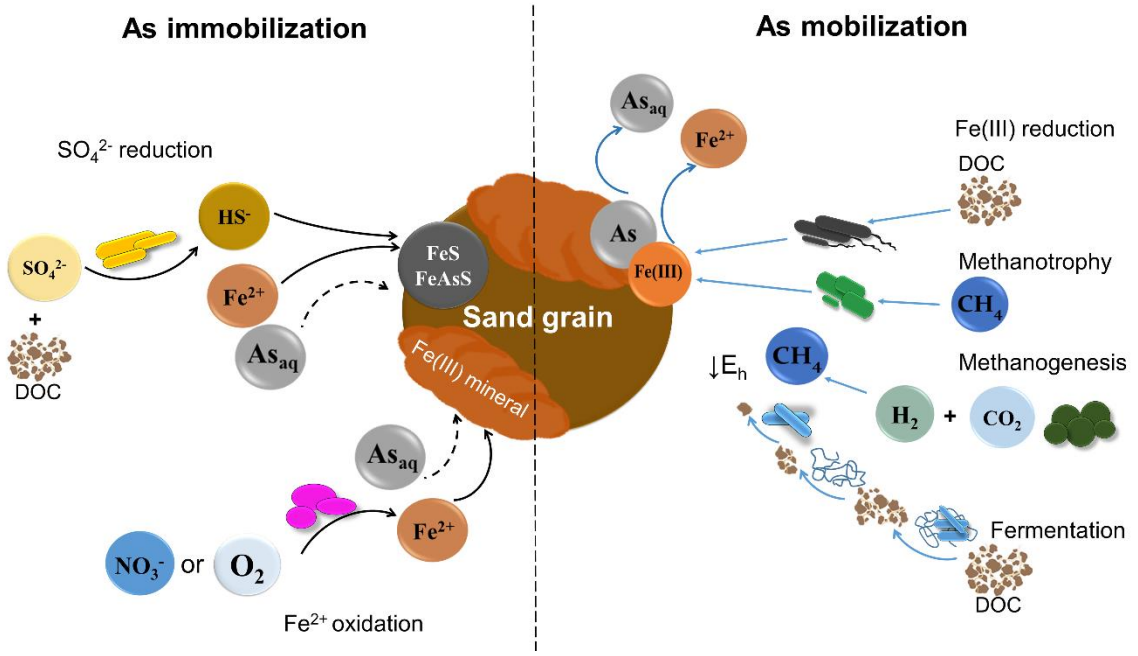

Figure 5.3 Overview of microbially mediated processes affecting As mobility in groundwater based on microbial community syudy in Van Phuc (Chapter 4).

Although taxa known to be involved in Fe(III) reduction such as *Geobacter*, *Bacillus*, *Deferribacteres*, *Thermincola*, *Geothrix* and *Magnetospirillum* were ubiquitous across the whole field site, the relative abundance of these species was rather low in most samples analyzed. As it was discussed in subchapter 5.1, it is probable that many other unknown taxa from the laboratory studies are also involved in Fe(III) reduction; however, the identity of these species is still unknown. It is also very likely that other groups of microbes, such as methanotrophs that are also very abundant in Van Phuc, can in fact mediate Fe(III) mineral

reduction and As mobilization (Chapter 4). Nevertheless, reductive dissolution must still be the dominating process for As mobilization, which is confirmed by strongly reducing conditions and high As and Fe concentration in Holocene groundwater aquifers. Finally, high concentration of NH_4^+ and a high abundance of microorganisms involved in nitrogen (N) cycling suggests that anaerobic oxidation of NH_4^+ coupled to Fe(III) reduction (Feammox) may take place in Van Phuc. In fact, Xiu et al. (Xiu et al., 2020) proposed Feammox to be one of the mechanisms of As release in the western Hetao Basin. The implications of the N cycle on As (im)mobilization deserve further investigations.

5.4 Final considerations

Water is the major component of the human body and it accounts for up to 60% of an adult person's weight (Mitchell, H.H. et al., 1945). Every organ of the human body contains a significant amount of water (brain and heart - 73%, skin - 64%, muscles and kidneys - 79%, bones - 31%) making it vital for life (Mitchell, H.H. et al., 1945). An average adult person can survive only a few days (from 2 to 7 days) without water. Therefore, regular supplies of about 1.5 L water per day is required for human organism to function properly (Jéquier & Constant, 2010). In regard to that, it is not surprising that water is deemed as the most essential natural resource (UNESCO, 2009). However, the importance of this precious resource is often underestimated and taken for granted. In consequence water resources are currently under huge pressure. Beside geogenic water contaminations such as described in this PhD thesis (i.e. As), freshwater systems are constantly threatened by human activities. Agriculture and aquaculture are the main contributors negatively affecting the water quality due to the intensive use of fertilizers, manures, pesticides and inefficient irrigation practices. Further threats come from industrial heavy metals pollutions, domestic untreated sewage, mining industry and recreational activities (Khatri & Tyagi, 2015). Future generations will have to face yet another danger threatening water supplies of which consequences are still largely unknown – the climate change. Water stress will continue to increase as a drastic decline in rainfall is expected, particularly in arid and semiarid areas (Misra, 2014). Number of studies showed that increasing salinization of surface and near-surface water is expected as an effect of climate change. It was estimated that more than 25 million people of the mega-deltas regions of Vietnam, Bangladesh and India are at risk of drinking ‘saline’ water (Hoque et al., 2016). Following the WHO by 2025, half of the world’s population will be living in water-stressed areas. As a result of decreasing access to safe and clean water, many people think that while the 20th century's wars were fought over oil, the 21st century’s conflicts will be fought over water (Selby, 2005).

Therefore, protection and sustainable use of this essential for our life water resources should be a priority for every and each of us.

Access to the safe drinking water might seem like a basic service, yet it is still a privilege available to the wealthiest nations that can afford efficient water treatment facilities, while over 1 billion people worldwide lack this service and use unsafe surface and groundwater sources (Sobsey et al., 2008). Scientific communities from all over the world make tremendous efforts to improve our knowledge and provide technology to address problems that developing countries are facing. I hope this PhD thesis contributes to these efforts. Explaining microbial mechanisms and understanding biological processes that affect behavior of As in groundwater is a base to find solutions and prevent future contamination.

5.5 References

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Statement of Personal Contribution

The work described in this PhD Thesis was founded by Deutsche Forschungsgemeinschaft (DFG) (KA 1736/41-1) and was a part of interdisciplinary and international project entitled "**Retardation and mobilization of arsenic at redox fronts under advective flow conditions - a concerted multidisciplinary approach (AdvectAs)"**. Information about the project can be found at http://gepris.dfg.de/gepris/projekt/320059499?language=en. Goals of this PhD Thesis could not be achieved without close collaboration with AdvectAs project members which are listed below:

Karlsruhe Institute of Technology - Institute of Applied Geosciences: M. Schneider, Dr. E. Eiche, Prof. Dr. A. Kontny, Prof. Dr. T. Neumann

University of Tübingen - Department of Geosciences: M. Glodowska, Prof. Dr. A. Kappler, Jun.-Prof. Dr. S. Kleindienst, Prof. Dr.-Ing. O. A. Cirpka, Dr. B. Rathi

Eawag, Swiss Federal Institute of Aquatic Science and Technology – Department of Water Resources and Drinking Water: A. Lightfoot, Dr. E. Stopelli, Prof. Dr. M. Berg, Prof. Dr. R. Kipfer, Prof. Dr. L. Winkel

Vietnam National University, Hanoi-Center for Environmental Technology and Sustainable Development (CETASD): Prof. Dr. Pham Hung Viet, Dr. Trang Pham, D. Vu, Dr. Vi Mai Lan, Mai Tran, Viet Nga

The University of Western Australia: Dr. Henning Prommer

The conceptual background of this PhD project (Module 2 of AdvectAs project: microbiology) was designed by Prof. Andreas Kappler and Jun.-Prof. Sara Kleindienst. However, the focus of Module 2 changed in the course of the work as new results were collected during the first sampling campaign in 2017 and new hypotheses were formulated by myself and Prof. Andreas Kappler. Prof. Andreas Kappler was the main supervisor throughout the project and Prof. Michael Berg was the second supervisor. Jun.-Prof. Sara Kleindienst was an unofficial supervisor and mainly involved in the work that realted to molecular microbiology and microbial ecology.

Unless otherwise stated, the experiments were either conceptualized by myself or together with Prof. A. Kappler. The experiments were conducted by myself. The discussion and analysis of the obtained results, as well as writing of all manuscripts were completed by myself with

support of Prof. A. Kappler; for chapters 2, 3 and 4 also in collaboration with Dr. Emiliano Stopelli, Dr. Daniel Straub, Prof. Michael Berg and Jun.-Prof. Sara Kleindienst. All AdvectAs project members were directly or indirectly involved in this work by sharing data, ideas, discussing results and brainstorming. In detail, the contributions of the named people including myself, as well as other people are as stated below:

Field Work: The field work required extensive planning and preparation in which all AdvectAs members were involved. The 2017 and 2018 sampling campaign was mainly organized under supervision of Prof. Michael Berg in a close collaboration with Vietnamese colleagues from Vietnam National University in Hanoi. Both sampling campaigns involved rotary drilling and piston coring for sediment collection and groundwater sampling for water analysis. All project members were assisting and helping in collecting samples for respective modules and both smelling campaigns were very successful because of efficient and productive collaboration.

Chapter 2: The experiment was conceptualized and designed by myself with support of Prof. A. Kappler, setup and conducted by myself. The data collection was carried out by myself. The ICP-MS analysis was performed by Dr. Emiliano Stopelli. The bioinformatic treatment of 16S rRNA gene sequences was done by Dr. Daniel Starub. The organic matter analysis was performed by Dr. Heike Knicker (^{13}C-NMR) and Dr. Julie Tolu (Pyrolysis-GC/MS). The discussion and analysis of the obtained results were made by myself and Prof. A. Kappler and members of AdvectAs project. The manuscript was written by myself with the support of Prof. A. Kappler and revised by all co-authors.

Chapter 3: The original hypothesis was formulated by myself and Prof. Andreas Kappler supported by the field data obtained from Alex Lightfoot and Prof. Rolf Kipfer. I designed the experiment, collected the samples, interpreted results and wrote the manuscript with supervision of Prof. Andreas Kappler. The ICP-MS analysis was performed by Dr. Emiliano Stopelli. Prof. Michael Berg organized sampling campaigned and contributed to the field data interpretation. Dr. Bhasker Rathi helped with the field data evaluation and geochemical calculations. Dr Daniel Straub conducted 16S rRNA sequencing data processing and bioinformatics while Jun.-Prof. Sara Kleindienst and Prof. Mike Jetten helped to interpret date and provided support with molecular microbiology. The manuscript was written by myself with the support of Prof. A. Kappler and revised by all co-authors.

Chapter 4: The work on Chapter 4 was conducted in a close collaboration with Dr. Emiliano Stopelli who is also equally contributing authors. The hypothesis was formulated by myself with the support of Dr. Emiliano Stopelli. The molecular microbiology data collection was

carried out by myself while hydrogeochemical data was collected by Dr. Emiliano Stopelli. The bioinformatics treatment of 16S rRNA gene sequences was performed by Dr. Daniel Starub. The manuscript was written by myself with the support of Dr. Emiliano Stopelli, Jun.-Prof. Sara Kleindienst, Prof. Michael Berg and Prof. Andreas Kappler, and it was revised by all co-authors.

I state hereby that I have not plagiarized or copied any of the text. Chapters 3 and 4 have been submitted to different scientific journals and they might be published with small variations elsewhere in the future.

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