



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

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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 CH₄ concentrations, methanotrophs thrive across the Holocene aquifer and the redox transition zone. Finally, our results underline the role of SO₄²⁻-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.

1. 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 (Postma et al., 2007; Dowling et al., 2002; Acharyya et al., 2000; Berg et al., 2007). Reducing conditions in these aquifers led to the mobilization and enrichment of As in groundwater (Charlet and Polya, 2006; Smedley and

Kinniburgh, 2002). Moreover, these regions are among the most densely populated areas on the planet (Smedley and 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 (Nitzsche et al., 2015; Berg et al., 2006). 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, chronic exposure to As has led to massive poisoning throughout local communities, manifesting as dermal lesions, cardiovascular diseases,

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and various types of cancer (Karagas et al., 2015; Smith et al., 1992).

Many mechanisms for the release of As into groundwater have been proposed (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 (Islam et al., 2004, 2005a; Chatain et al., 2005). 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. (Islam et al., 2005a), *Shewanella* (Cummings et al., 1999), or *Geothrix* sp. (Islam et al., 2005b), but also underline the importance of carbon quantity, quality, and bioavailability as necessary fuels for Fe(III) mineral reduction (Chatain et al., 2005; Neumann et al., 2014; Duan et al., 2008; Héry et al., 2010; Glodowska et al., 2020). 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) (Cummings et al., 1999; Islam et al., 2005b; Neumann et al., 2014; Duan et al., 2008). 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 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 and 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 (Katsoyiannis and Zouboulis, 2006; Hohmann et al., 2010). 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 (Ike et al., 2008; Garcia-Dominguez 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) (Newman et al., 1997; Bostick and Fendorf, 2003). 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 (Stucker et al., 2014; Kumar et al., 2016; Guo et al., 2016). 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 (Postma et al., 2007; Harvey et al., 2002; Jessen et al., 2008; Liu et al., 2009; Polizzotto et al., 2005; Postma et al., 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 and 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

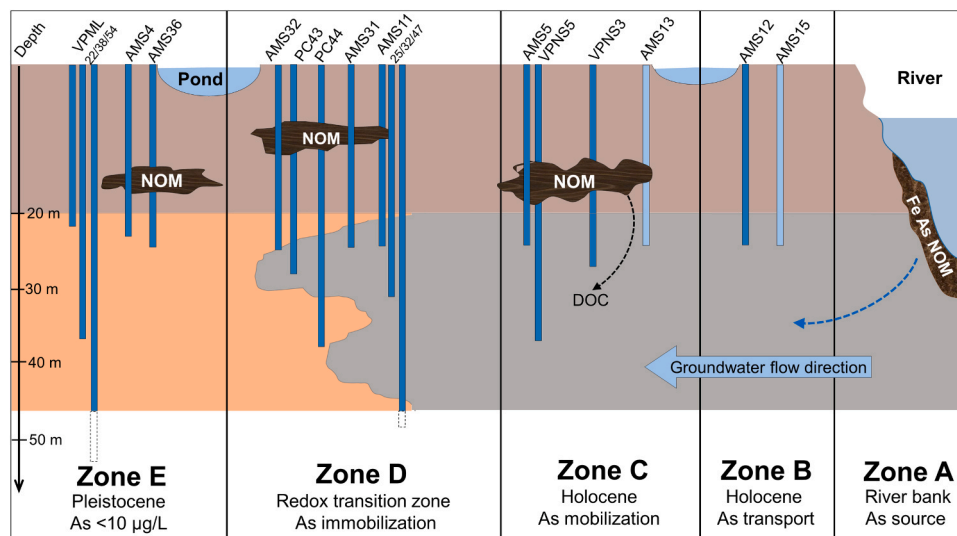


Fig. 1. Two-dimensional cross-section of Van Phuc aquifers divided by hydrogeochemical zone (A–E), as reported in Stopelli et al. (2020), presenting the distribution of monitoring wells across the transect. Lighter blue wells are in the proximity to the transect within comparable hydrogeochemical zones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 gray 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. Several previous studies investigated the hydrology, geology, lithology and mineralogy of this site (Eiche et al., 2008; Stopelli et al., 2020; van Geen et al., 2013; Eiche et al., 2017; Wallis et al., 2020). Moreover, we have recently provided a detailed description of the site, including the spatial and temporal evolution of As concentrations in the context of hydrogeochemical conditions (Stopelli et al., 2020). Yet, none of these studies assessed the role of microbial communities in As cycling in situ.

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.

2. Material and methods

2.1. 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). An important feature of the site is an inverted 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 (Fig. 1), as described in 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 SO₄²⁻-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 Fig. 1).

2.2. 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 O₂, 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 NH₄⁺ and ortho-PO₄³⁻ 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 O₂, 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.

2.3. 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. (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 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 75 °C for 30 s. This was followed by a final elongation step at 72 °C 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.

2.4. 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 (Straub et al., 2020). 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 et al., 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).

2.5. Statistical analysis

All statistical analyses were carried out in R (v3.4.4) (R Core Team, 2018). The Bray–Curtis dissimilarity (Sorensen et al., 1948), calculated via QIIME2, was plotted using Non-metric Multidimensional Scaling (NMDS) via phyloseq (McMurdie and 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 and Hochberg, 1995).

2.6. 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 (*arrA*) 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 S1. Quantitative PCRs on DNA extracts obtained as described above were performed in triplicate using Sybr-Green® Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on the C1000 Touch thermal cycler (CFX96™ real time system). Each quantitative PCR assay was repeated three times, with triplicate measurements calculated for each sample per run. Data analysis was done

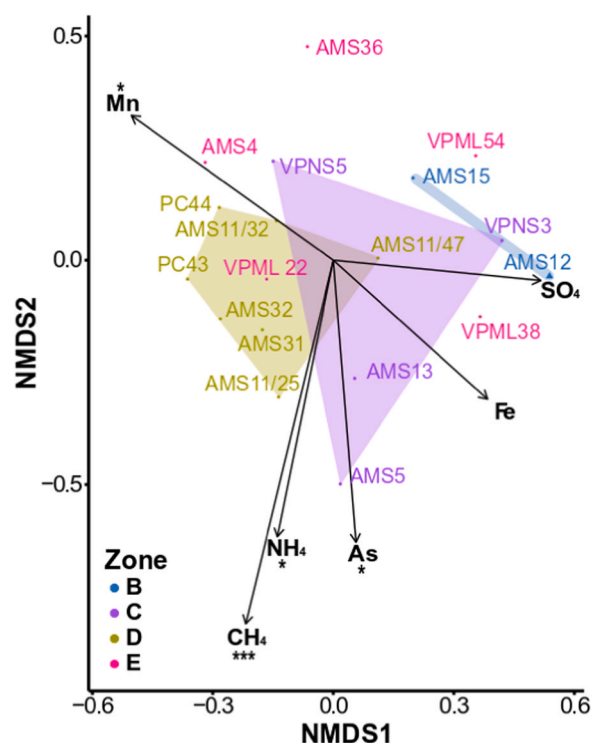


Fig. 2. Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarity (stress = 0.17) to visualize the main biogeochemical groundwater parameters (arrows for As, Fe, CH₄, SO₄²⁻, NH₄⁺, and Mn) associated with the microbial community composition. The strength of the interaction is shown by the length of the arrows; significant correlations are shown for As (*, *p* < 0.03), CH₄ (***, *p* < 0.001), NH₄⁺ (*, *p* < 0.01) and Mn (*, *p* < 0.05).

using the Bio-Rad CFX Maestro 1.1 software version 4.1 (Bio-Rad, 2017).

3. 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), CH₄ (*p* = 0.001), NH₄⁺ (*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 (Fig. 2). Non-metric Multidimensional Scaling (NMDS) resulted in the grouping of the wells, which largely reflected the hydrogeochemical zonation proposed by 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.

3.1. 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 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 (Wallis et al., 2020; Stahl et al., 2016). This is due to the constant supply of sediments rich in bioavailable C and reactive Fe(III) (oxyhydr)oxides from the Red

Table 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	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	0.26	< 0.25	4.3	< 0.25	< 0.25	4.3	6.2	1.7	< 0.25	< 0.25
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	< 0.5	< 0.5	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	< 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	< 0.1	6.0	0.5	0.4
Fe	mg/L	0.05	< 0.05–14	12	0.54	14	12	20	14	13	< 0.05	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	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	1.5	< 0.1	< 0.1	1.5	2.3	0.6	< 0.1	< 0.1
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	< 0.04	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	–	< 0.13	< 0.13	53	5	1.9	30	51	< 0.13	< 0.13	28	< 0.13	25	15	< 0.13	< 0.13	< 0.13	< 0.13	< 0.13

* 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.

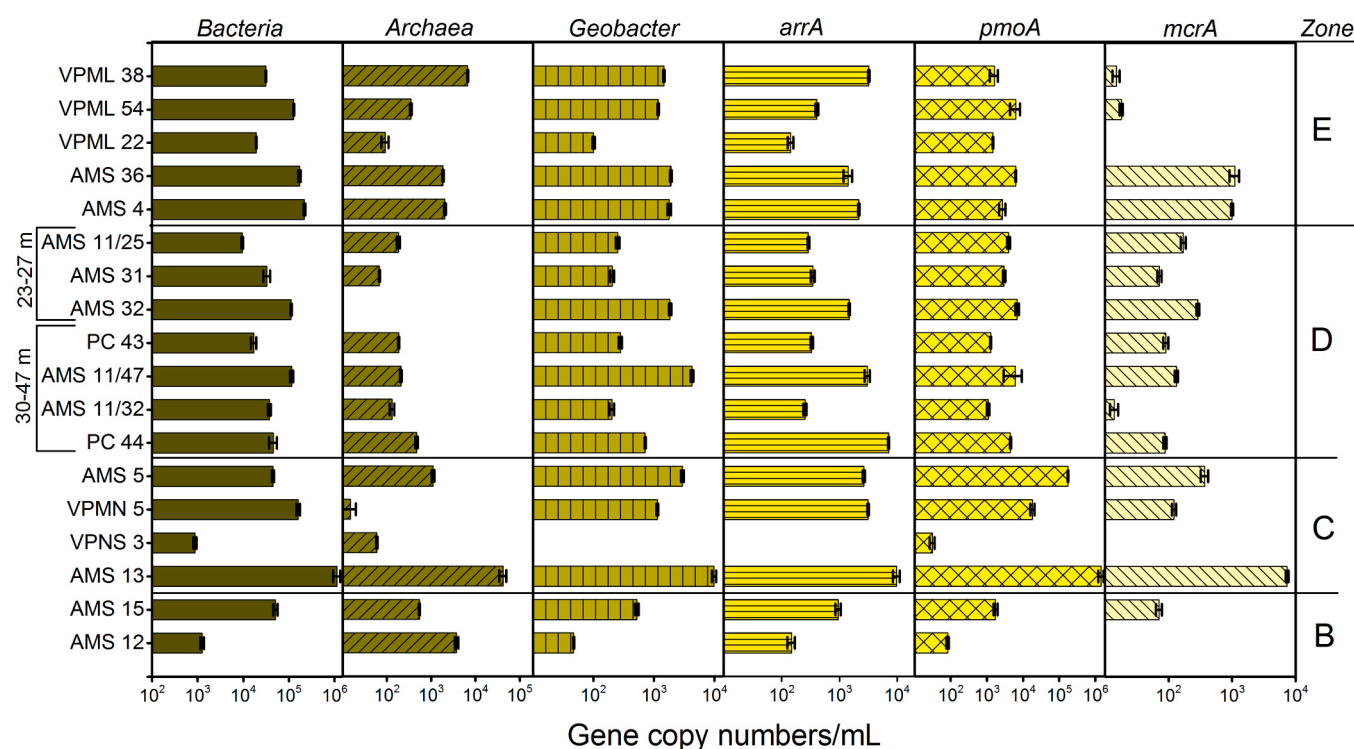


Fig. 3. DNA-based quantitative PCR analysis of bacterial 16S rRNA genes, archaeal 16S rRNA genes, *Geobacter* specific 16S rRNA genes, arsenate reductase genes (*arrA*), particulate methane monooxygenase genes (*pmoA*), and methyl-coenzyme M reductase subunit alpha genes (*mcrA*) in the groundwater wells of different zones (B–E) of the Van Phuc aquifer. Error bars show standard deviation from three measurements.

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, Fig. 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 (Fig. 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 S2), 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.

Among the active microbial community (Fig. 4), fermenters appeared to be the most abundant group of microorganisms, with the majority of taxa related to *Firmicutes*, *Chloroflexi*, and *Bacteroidetes* (Kampmann et al., 2012; Gupta et al., 2014). In addition, predicted fermentation dominated among all environmentally relevant metabolic pathways, as inferred from 16S rRNA amplicon sequences (Fig. 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 (McMahon and Chapelle, 1991; Chapelle, 2000). 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). Low redox potential was shown to play an important role in As dynamics, generally favoring the release of As associated with Fe(III) minerals (Shaheen et al., 2016; LeMonte et al., 2017; Frohne et al., 2011). 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 *Thermodesulfobionia* (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 and Fendorf, 2003; Keimowitz et al., 2007; Huerta-Diaz et al., 1998; 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 (Kirk et al., 2004; Bostick 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.

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%

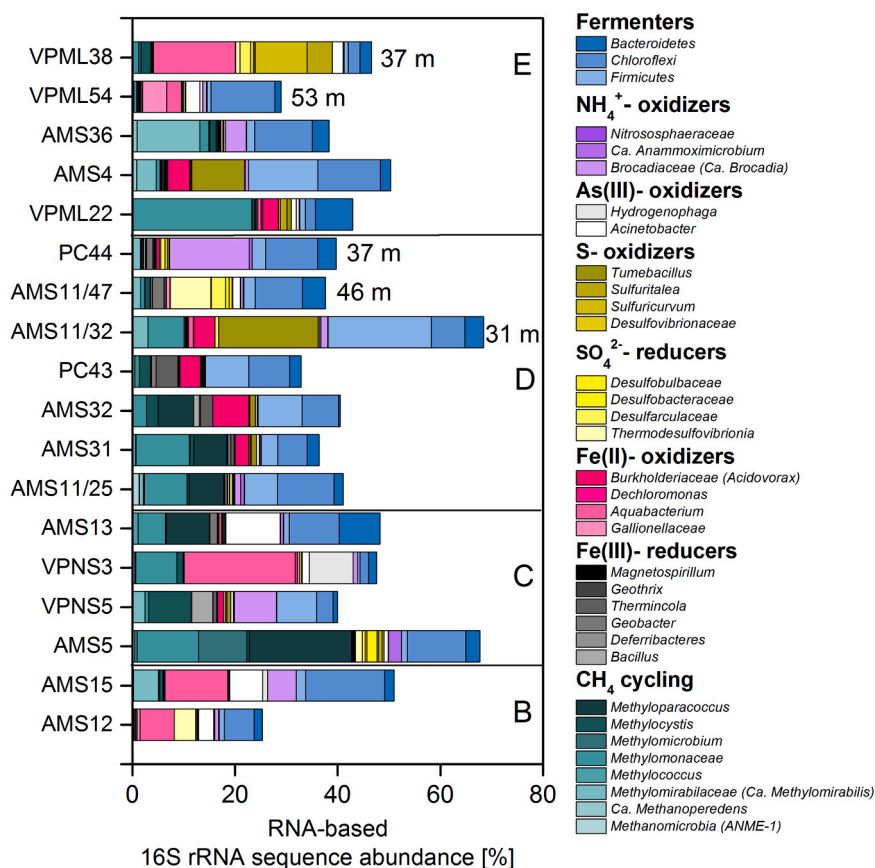


Fig. 4. RNA-based 16S rRNA sequence abundance of selected taxa (including potential function) in groundwater samples of Van Phuc. Wells are divided into several zones according to Stopelli et al. (2020): B: As-transport, C: As-mobilization, D: As-retardation, E: As-pristine/retardation. The wells had a depth between 20 and 27 m, except for those marked otherwise.

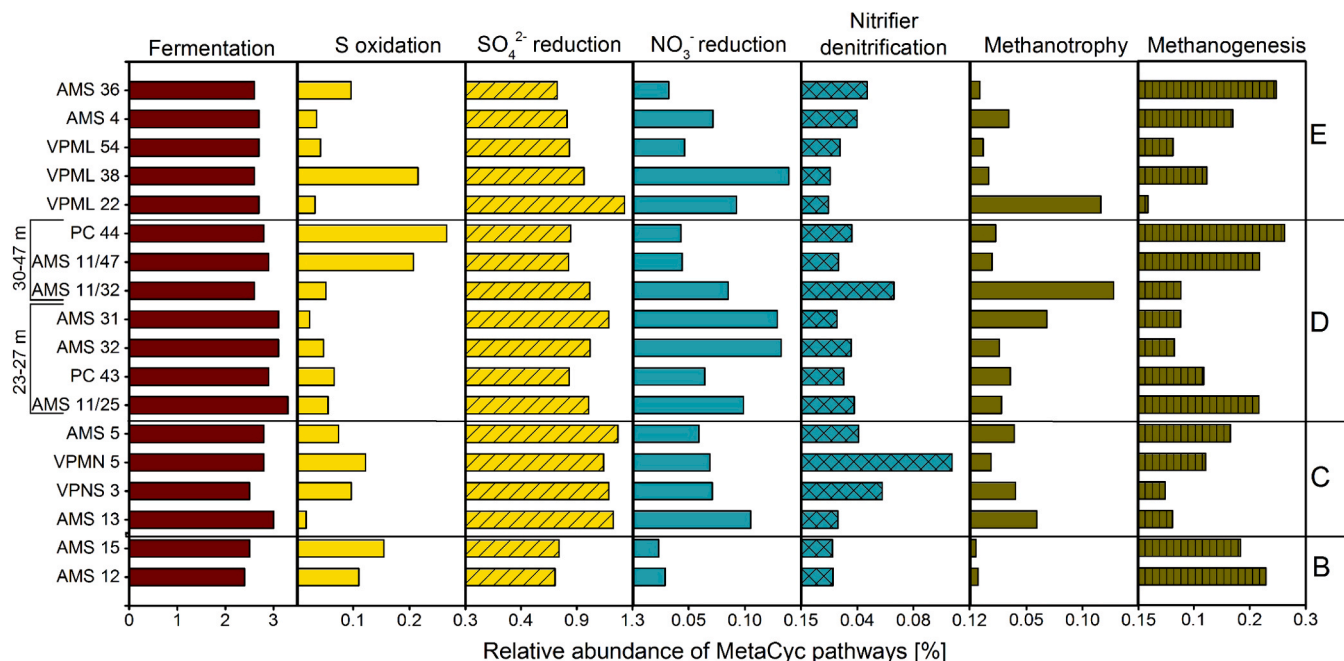


Fig. 5. Relative abundance of predicted environmentally relevant microbial metabolic pathways in groundwater wells divided by zone (B–E). Metabolic potential was inferred from RNA-based 16S rRNA amplicon sequencing data and is based on MetaCyc pathways.

in AMS15). Different strains belonging to the genus *Aquabacterium* were shown to be capable of Fe(II) oxidation (Zhang et al., 2017; Kalmbach et al., 1999). Moreover, in many previous studies focusing on As-contaminated aquifers, *Aquabacterium* was found abundantly (Sutton et al., 2009; Li et al., 2014, 2013), 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). These microorganisms have been found to be very efficient in As removal from contaminated soil (Karn and Pan, 2016), and it is therefore possible that these bacteria also contributed to reducing As concentrations in groundwater sites from Van Phuc.

No CH₄ was detected in zone B and, in agreement with this finding, not many microorganisms related to methane cycling were present in the groundwater samples (Fig. 4). Coherently, *mcrA* and *pmoA* genes related to methane cycling were also less abundant in these wells when compared to other zones (Fig. 3).

To summarize zone B, carbon degradation and fermentation likely lead to As mobilization, while SO₄²⁻ 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.

3.2. Zone C: methanogenic Holocene aquifer—further As mobilization

Four wells in the Holocene aquifer in zone C exhibited measurable CH₄ 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 1). Moreover, in this zone, increased DOC values were present (2.3–8.5 mg/L) along with NH₄⁺ (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 (Fig. 4). In addition, relatively high *Geobacter* (up to $9.8 \times 10^3 \pm 7.7 \times 10^2$ /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 (Fig. 3, Table S3). 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% (Fig. 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 CH₄ concentrations (up to 53 mg/L) (Table 1). The presence of high concentrations of CH₄ in zone C is in agreement with a high abundance of diverse microbial taxa related to the CH₄ cycle, among which *Methyloparacoccus* (20%), *Methylomonaceae* (13%), *Methylomicrobium* (9.5%), and *Methylomirabilaceae* (2.5%) were found to be dominating (Fig. 4). These results are further supported by high *pmoA* and *mcrA* gene copy numbers in this zone (Fig. 3).

Within the highly reducing conditions of zone C, putative As(III)-oxidizers, such as *Acinetobacter* (Karn and Pan, 2016) and *Hydrogenophaga* (vanden Hoven and Santini, 1966), were also abundant (up to 10%) (Fig. 4). These microorganisms can cope with high concentrations of As due to detoxification mechanisms, where As(III) is oxidized by a periplasmic 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, the behavior of As is predominantly controlled by reductive processes that outcompete oxidative ones, since As concentrations in Holocene reach 300–500 µg/L. The relation between increased CH₄ 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.

3.3. 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), NH₄⁺ (15–25 mg/L), and CH₄ (15–51 mg/L) concentrations. Deeper wells (30–37 m depth), however, had no As, no CH₄, rather low NH₄⁺ (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.

The shallow wells AMS31, AMS32, PC43, and AMS11/25 (23–27 m deep) 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 CH₄ and NH₄⁺ 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 CH₄ 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; Fig. 4). Although the relative abundance of methanotrophs was lower in zone D compared to zone C, higher rates of CH₄ 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 CH₄ may serve as an electron donor for anaerobic methanotrophs that couple CH₄ oxidation with Fe(III) reduction (Ettwig et al., 2016; Aromokeye et al., 2020; Cai et al., 2018). In fact, our latest study (Glodowska et al., in press) 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 CH₄ oxidation can also be coupled with SO₄²⁻ reduction, a process driven by the syntrophic consortia of methanotrophic archaea (ANME-1, ANME-2a,b,c, and ANME-3) and SO₄²⁻-reducing bacteria (Boetius et al., 2000; Orphan et al., 2001; Scheller et al., 2016; Knittel and Boetius, 2009; Milucka et al., 2012), which can potentially contribute to As immobilization via precipitation. Finally, a recent study by Leu et al. (2020) revealed that CH₄ 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 and Morgan, 1995) and they can retain As in sediments; however, once Mn(IV) gets reduced to dissolved

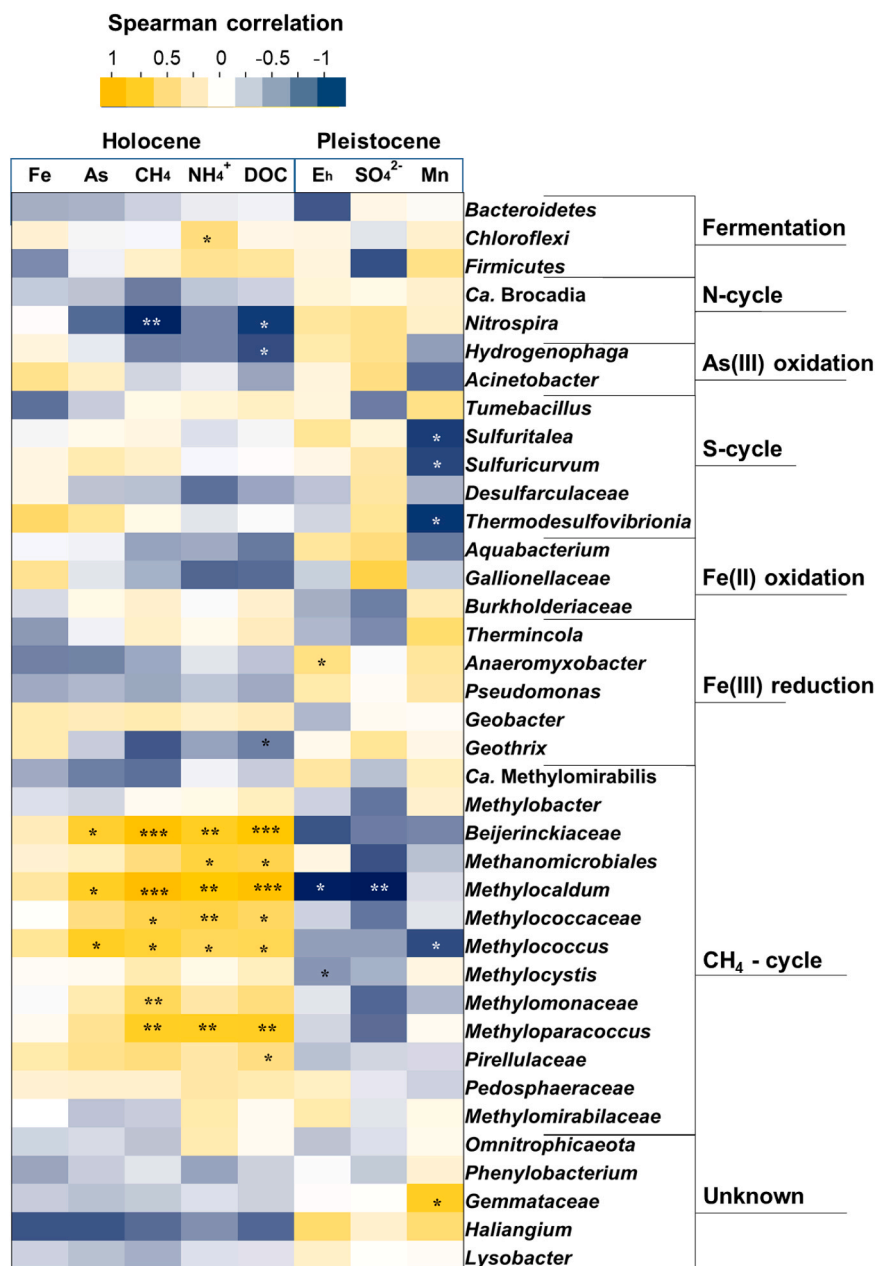


Fig. 6. Heatmap of Spearman rank correlations of microbial taxa with hydrogeochemical parameters, such as dissolved Fe, As, CH₄, NH₄⁺ and DOC (elevated concentrations more characteristic of Holocene aquifers) and Eh, SO₄²⁻ and Mn (elevated concentrations more characteristic of Pleistocene aquifers), with the most abundant taxa clustered by their putative functions. Significance levels (Benjamini–Hochberg corrected): $p < 0.1$ (*), $p < 0.05$ (**), $p < 0.001$ (***).

Mn²⁺, As can be released into the groundwater. Therefore, CH₄-rich groundwater flowing through Pleistocene sediments containing Fe(III) and Mn (III/IV) (oxyhydr)oxides as well as the presence of SO₄²⁻, can create favorable conditions for anaerobic CH₄ oxidation in the redox transition zone. These hydrogeochemical conditions, together with abundant methanotrophs, strongly imply that complex biogeochemical interactions between CH₄ and As are likely taking place in zone D. Considering the higher abundance of Fe(III) compared to Mn (III/IV) and SO₄²⁻, 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).

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, SO₄²⁻-reducing bacteria, such as *Thermodesulfovibronia* (8%) and *Desulfarculaceae* (3%), were abundant (Fig. 4). In agreement, the predicted S-oxidation pathway appeared to be more abundant at deeper parts of zone D (Fig. 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 1). Additionally, framboidal pyrites and other iron sulfide minerals such as mackinawite were previously observed in sediments (Kontny et al., unpublished), further implying that active S-cycling is taking place at the redox transition zone.

The composition of the in situ active microbial community (Fig. 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 (Sutton et al., 2009; Straub et al., 2004), 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). 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 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; Sørensen et al., 2018).

3.4. Zone E: Pleistocene pristine aquifer—As immobilization

Dissolved As concentrations generally remained below the WHO guideline value of 10 $\mu\text{g/L}$ 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 (< 0.1–1 $\mu\text{g/L}$), and Fe (0.07–0.75 mg/L) as well as dissolved CH_4 below the detection limit (Table 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 (Fig. 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 and 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 (Fig. 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 $\mu\text{g/L}$; Table 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%) (Fig. 4) as well as a trace concentration of SO_4^{2-} (0.26 mg/L) (Table 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.

3.5. 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 (Fig. 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 (Fig. S1). As a result, a wide range of short-chain fatty and organic acids are likely produced, including acetate, lactate, formate, or propionate (McMahon and Chapelle, 1991; Chapelle, 2000). 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 (Postma et al., 2007; Dowling et al., 2002; Liu et al., 2009; Sutton et al., 2009), 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 and 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 *mcrA* 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 60% 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:



and second, hydrogenotrophic methanogenesis:



where CO_2 is reduced to CH_4 (Govert and Conrad, 2009). Metabolic pathways inferred from 16S rRNA amplicon sequences (Fig. 5) suggested that the predicted acetoclastic methanogenesis is dominating in Van Phuc aquifers (Fig. S2).

3.5.1. 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-} (Aromokeye et al., 2020; Cai et al., 2018; Boetius et al., 2000; 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 (Ettwig et al., 2016; Aromokeye et al., 2020; Cai et al., 2018), a process that we confirmed within Van Phuc sediments and that can lead to significant As mobilization (Glodowska et al., in press). Furthermore, microorganisms with the metabolic potential for CH_4 oxidation were abundantly present in most of the sampled wells (Figs. 3 and 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^+ (Fig. 6). Some of these taxa are also significantly positively correlated with As, e.g. *Methylococcus* ($r = 0.65$), *Methylocaldum* ($r = 0.64$) or *Beijerinckiaceae* ($r = 0.63$) (Fig. 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 (Fig. 6).

3.5.2. Low abundance of known Fe(III)-reducers

Active microbial taxa known to be involved in dissimilatory Fe(III) reduction, such as *Bacillus*, *Deferribacteres*, *Geobacter*, *Thermicola*, *Geothrix*, and *Magnetospirillum* were ubiquitous across the whole field site, although at rather low relative abundances (Fig. 4). Many previous studies have shown the importance of Fe(III)-reducing bacteria in As mobilization (Islam et al., 2005a, 2005b; Héry et al., 2008; 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; Li et al., 2013; Kim et al., 2012). 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.

3.5.3. 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 (Fig. 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 (Fig. 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 (Fig. 6).

3.5.4. Cryptic N-cycle

Interestingly, pathways for predicted nitrate (NO_3^-) reduction and nitrifier denitrification were also inferred in all wells (Fig. 5). At the same time, active microorganisms involved in the N-cycle were identified as NH_4^+ -oxidizers in some of the wells (Fig. 4), which are mostly associated with Pleistocene-like moderate reducing conditions (Fig. 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 and 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. (2020) proposed Feammox to be one of the mechanisms involved in As release in the western Hetao Basin.

3.5.5. 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 (Fig. 2 and Fig. 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 (Fig. 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. 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 (Fig. 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. We believe that our observation can be widely transferable and helps to understand As behavior in the context of microbially mediated processes in similar aquifers in South and Southeast Asia, including the other young Holocene aquifers of the Red River Delta.

editing, Funding acquisition.

Declaration of Competing Interest

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

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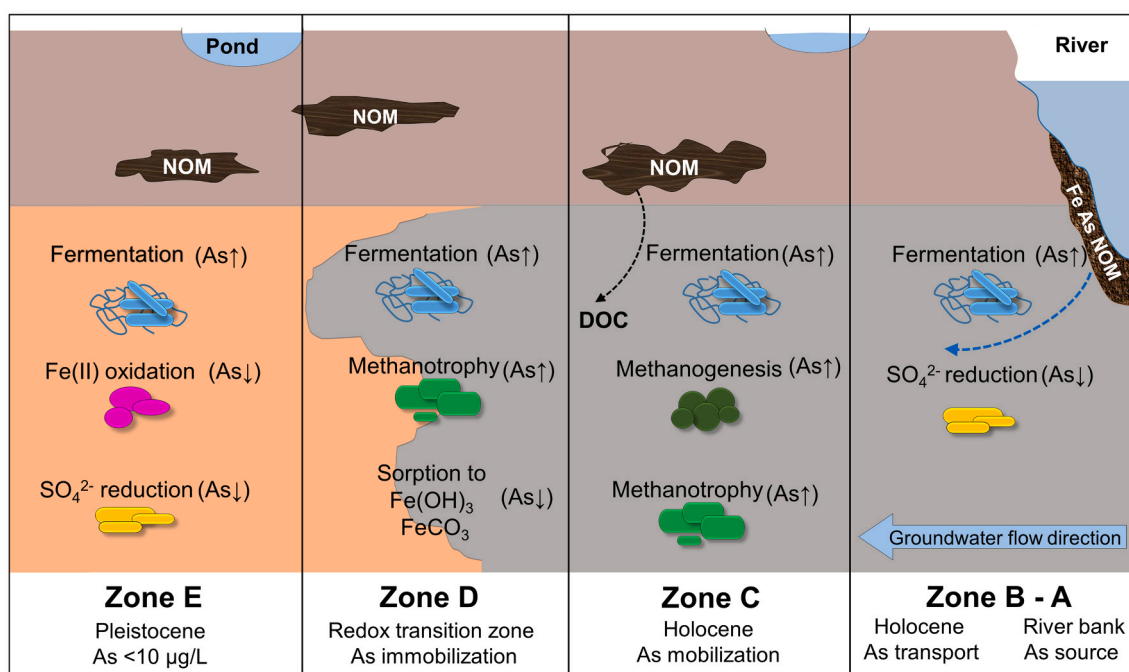


Fig. 7. Two-dimensional cross-section of Van Phuc aquifers divided by hydrogeochemical zone (A–E), as reported in Stopelli et al., 2020. For each zone, the main microbial processes are indicated together with their net effect on As mobilization ($\text{As}\uparrow$) or immobilization ($\text{As}\downarrow$).

CRediT authorship contribution statement

Martyna Glodowska: Conceptualization, Investigation, Writing - original draft, Writing - reviewing & editing, Visualization. **Emiliano Stopelli:** Conceptualization, Investigation, Writing - reviewing & editing. **Daniel Straub:** Data curation, Formal analysis. **Duyen Vu Thi, Pham T.K. Trang, Pham H. Viet, AdvectAs team members:** Resources, Project administration. **Michael Berg:** Supervision, Writing - reviewing & editing, Project administration, Funding acquisition. **Andreas Kappler:** Supervision, Writing - reviewing & editing, Funding acquisition. **Sara Kleindienst:** Supervision, Writing - reviewing &

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2020.124398](https://doi.org/10.1016/j.jhazmat.2020.124398).

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