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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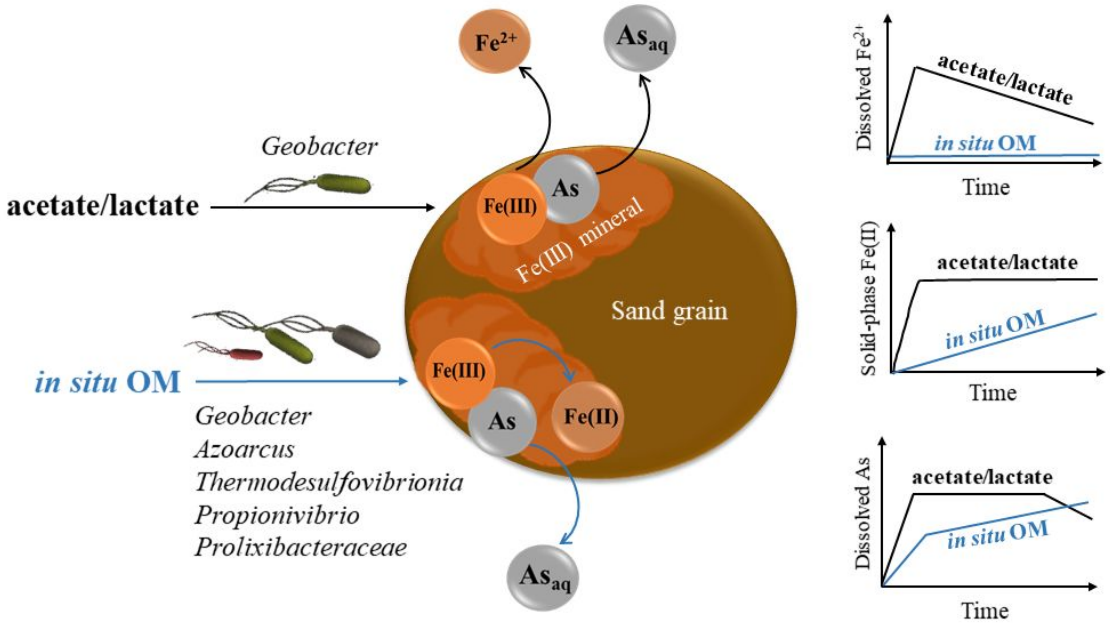
Abstract

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.

Keywords: As mobilization, *in-situ* organic matter, Fe(III) mineral reduction

Graphical abstract



INTRODUCTION

Arsenic (As) is a toxic metalloid that causes serious health issues such as arsenicosis, cardiovascular disease and increased risk of cancer[1,2]. 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[3]. Southeast Asia is a particularly affected part of the world[4]. 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[3]. Although our knowledge about processes affecting As mobilization has increased substantially in recent years[5,6], 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[7–10]. Organic matter (OM) plays a key role in this process, in particular as electron donor for microorganisms[11–14]. 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[12,15–20]. 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[12,21]. Additional organic compounds that have been tested in such studies are humic substances or water from a drainage tube[22] and plant material such as ground bean leaves, barley straw or pine sawdust[23]. 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) [12,24,25]. These C sources can contain complex plant-based OM which is considered rather resistant to chemical and biological degradation[26,27] 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[16,28,29]. 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[30]. 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[30–32]. 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[33]. 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.

MATERIALS AND METHODS

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[30–34]. 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[34]. 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[35]. The total Fe and As contents of the 30-m sediment were determined by XRF (Bruker, AXS S4 Explorer).

Organic Matter Extraction and Characterization. The dominating type of C present in the aquifer originates from vascular C3 plants (mainly mangroves)[33]. 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)[36]. 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 S1) 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.

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[37]) with 5 mL (final volume) sterile synthetic groundwater medium supplemented with C (modified from Rathi et al.[38]; 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): 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).

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)[39]. 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[40]. 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[39]. 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.

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.[41]. Bacterial and archaeal 16S rRNA genes were amplified using universal primers 515f: GTGYCAGCMGCCGCGGTAA[42] and 806r:

GGACTACNVGGGTWTCTAAT[43] 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 S3).

RESULTS AND DISCUSSION

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[33,34]. 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 S1). 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 1A) were generally similar to each other (a certain similarity between OMC and OMS was also confirmed by similar EEM spectra, Fig. S2), 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[44], characteristic for polysaccharides. The peak at 1616 cm^{-1}, specific for aromatic C=C (alkene) and conjugated C=O or C=N[44], was more pronounced in the OMC spectrum, suggesting the presence of lignin-derivatives[45] 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[44]. 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[46].

In addition to FTIR, solid-state ^{13}C-NMR was applied to characterize the chemical properties of both types of extracted OM (Figure 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)[47], 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 S3), generally, the NMR intensity distribution of different C-functional groups in both extracted OMs and in the two bulk sediments (Figure 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 S4)[48,49]. 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. 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 S1C) 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[50], also indicating the presence of plant-derived OM[51]. 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[16,29].

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[52]. 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). However, the extent and rates of Fe(III) reduction and As mobilization differed between various C sources supplied. The highest concentration of Fe(II) in the sediments was recorded in A-/L-amended microcosms (Figure 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 (Figures 2B). In microcosms supplied with OMC and OMS Fe^{2+} was not released into solution.

316 Our data showed that in microcosms supplied with OMC and OMS, dissolved Fe^{2+} stayed at a
317 similar level as in the biotic and abiotic controls (CON+ and CON-) suggesting that the formed
318 Fe(II) remained as either sorbed Fe(II) or Fe(II) mineral in the sediments. The saturation index
319 for different minerals was calculated using PhreeqC in order to explain the lack of Fe^{2+} release
320 in microcosms supplied with *in-situ* OM (see Table S5). The calculation showed that no siderite
321 precipitation is expected. Therefore, the lack of Fe^{2+} mobilization could be due to adsorption of
322 Fe(II) on the remaining poorly crystalline Fe(III/II) minerals[53] or formation of NOM-Fe
323 complexes could have prevented Fe^{2+} mobilization. It was previously shown that some
324 functional groups such as carboxyl groups, which were also present in the extracted OM used in
325 our study, are particularly prone to create complexes with Fe(II) at neutral pH[54].
326 Quantification of dissolved As showed that trends in As mobilization did not fully correlate
327 with Fe(III) reduction in the sediments (Figure 2C). By the first 6 days of incubation dissolved
328 As was found to be higher in A-/L-amended setups than in OMS and OMC setups, where
329 almost 8 µg/L dissolved As was released from 1 g of sediment, i.e. a mobilization of 0.7% of
330 the total As present, compared to less than 4 µg/L As in OMS and OMC setups (the %-values of
331 mobilized As were calculated using dissolved As concentrations in the 5-ml-volume at given
332 time points divided by the sedimentary As content determined by XRF). The concentration of
333 dissolved As decreased after 60 days in A-/L-setups (to 6.3 µg/L at the end of incubation),
334 which might be related to the decrease of aqueous Fe^{2+}, possible formation of secondary Fe
335 minerals (that are not considered in our saturation index calculation, Table S5) and As co-
336 precipitation[55]. A similar rapid Fe(III) reduction and As mobilization followed by As
337 immobilization due to co-precipitation with secondary minerals has been shown for West
338 Bengal sediments amended with acetate[56] and glucose-/lactate-amended As-contaminated

339 soils[57]. Despite lower extents of Fe(III) reduction, ultimately (day 100) a higher As
340 concentration was recorded in the presence of OMC (8.3±0.3 µg As/L; *t*-test, $p<0.005$) and
341 OMS (8.8±0.8 µg As/L; *t*-test, $p<0.005$) compared to A/L setups, corresponding to mobilization
342 of 0.75 and 0.8% of the total As, respectively. On the one hand, this higher As concentration
343 despite lower Fe(III) reduction could be due to competitive sorption of the OM and As. It is
344 known that organic compounds such as citrate or humic acids can decrease adsorption of
345 phosphate to soil and to Fe(III) minerals such as goethite[58,59]. As(V) can be considered as an
346 analog of phosphate[60], and therefore OM could affect As(V) sorption, but also As(III) sorption,
347 through competition for reactive surface sites and could lead to desorption of As. On the other
348 hand, OM can change As speciation through redox reactions[61,62] and formation of binary and
349 ternary complexes with Fe and As[63]. Such dissolved NOM-As-Fe complexes can increase the
350 mobility of As, resulting in increased aqueous As concentrations in groundwater[62,64]. Overall,
351 our study demonstrated that *in-situ* OM (including OM from the aquitard that can potentially be
352 mobilized) can trigger microbial Fe(III) reduction and can contribute to As release. Although
353 initially (until 60 days of incubation) more As was present in solution in microcosms supplied
354 with OMC compared to OMS, the final As concentration (8 µg As/L) was the same for
355 microcosms amended with both types of OM. It has to be noted that although 8 µg As/L might
356 seem to be insignificant, the water to sediment ratio in our microcosms (5:1 wt/wt) was much
357 higher compared to the one in the aquifer (1:8 (wt/wt) assuming a porosity of 25% and a
358 sediment density comparable to quartz)[65]. Under these conditions the concentration of 8 µg
359 As/L from our experiment would be equivalent to a concentration of 352 µg As/L in the field.
360 Therefore, even considering that there are overall differences between laboratory and field
361 conditions regarding water flow, temperature, history of As release and local As accumulation,

exact identity of carbon used by microorganisms, etc., ~~This~~ our measured As concentration is similar to the concentration measured in contaminated Holocene groundwater at our field site in Van Phuc. This~~,~~ suggest~~sing~~ 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.

These~~is~~ observations potentially show~~s~~ 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.

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 >$3.0x10^6$±$3.5x10^5$ bacterial 16S rRNA gene copy numbers per g sediment within the first 10 days of the incubation (Figure 3A). However, A/L was quickly consumed leading to a decrease (ca. 90%) of the bacterial abundance to $2.4x10^5$±$5.0x10^4$ 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 $1.5x10^6$±$8.6x10^4$ bacterial 16S rRNA gene copy numbers per g sediment and doubled from $1.5x10^6$±$1.9x10^5$ to $3.4x10^6$±$4.7x10^5$ 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 $4.2x10^5$±$3.1x10^4$ to $1.8x10^6$±$1.6x10^5$ 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 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.

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 3C and 3D) that were previously detected in As contaminated environments[66,67]. The *arr*A gene was detected in all microcosms, although at one order of magnitude lower than bacterial 16S rRNA gene copy numbers (Figure 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 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 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 S6). *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)[68] were the most abundant group of microorganisms, representing >10% 16S rRNA relative gene sequence abundance. Other abundant taxa were *Moraxellaceae* (5%), potential arsenite-oxidizing[69] *Hydrogenophaga* (4%), and potential ammonia-oxidizing archaea[70] affiliating with *Nitrososphaeraceae* (3%). 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[11], 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*[71] 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. 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[72], 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[50] as well as in other As-contaminated aquifers where Fe(III) reduction is a significant terminal electron-accepting process[9,11,58,73], 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 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

477 at day 10. Although *Geobacter* also enriched in the presence of natural OM, other taxonomic
478 groups such as *Prolixibacteraceae*, *Erysipelothrix*, *Dechloromonas*, *Propionivibrio*,
479 *Desulfotomaculum*, *Azoarcus* and *Thermodesulfovibrionia* enriched as well. Some of these taxa
480 were previously reported to be present in As-contaminated environments, suggesting their
481 potential direct or indirect role in As cycling[71]. Therefore, our results demonstrate that using
482 bioavailable C such as acetate/lactate favors growth of specific microorganisms (i.e.
483 *Geobacter*). However, based on VFA analysis of the porewater, we know that acetate and
484 lactate can be found in the aquifer only sporadically and at concentrations below a few μM,
485 therefore these VFA are probably not the main carbon sources *in-situ*. In contrast, *in-situ* OM
486 enriched diverse taxa and maintained the microbial population for much longer (whereas in the
487 A-/L-amended setups, after 10 days when acetate was already consumed, the cell numbers
488 decreased drastically), suggesting that an increasing complexity of OM might stimulate more
489 diverse microbial communities for much longer in the groundwater aquifer, contributing to
490 slower but prolonged Fe(III) reduction and As mobilization.

491 **Environmental implications.** Our study demonstrates that the identity and reactivity of the
492 organic matter controls the rates and extent of Fe(III) reduction and subsequent As mobilization
493 from aquifer sediments under anoxic conditions. Although the commonly used easily
494 bioavailable C-sources such as acetate, lactate, glucose or lactose are useful as a proxy in simple
495 laboratory experiments, they do not fully represent environmentally relevant OM, particularly
496 when used at very high concentrations. In order to gain a full understanding of the prevalent
497 processes and the microbial community involved in the environment, it is necessary to compare
498 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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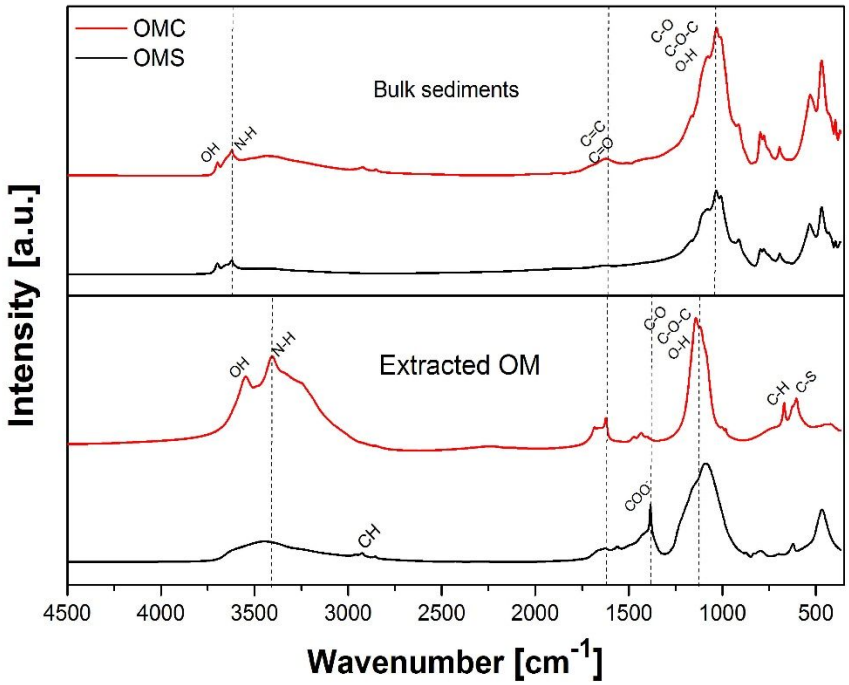
FIGURES

A



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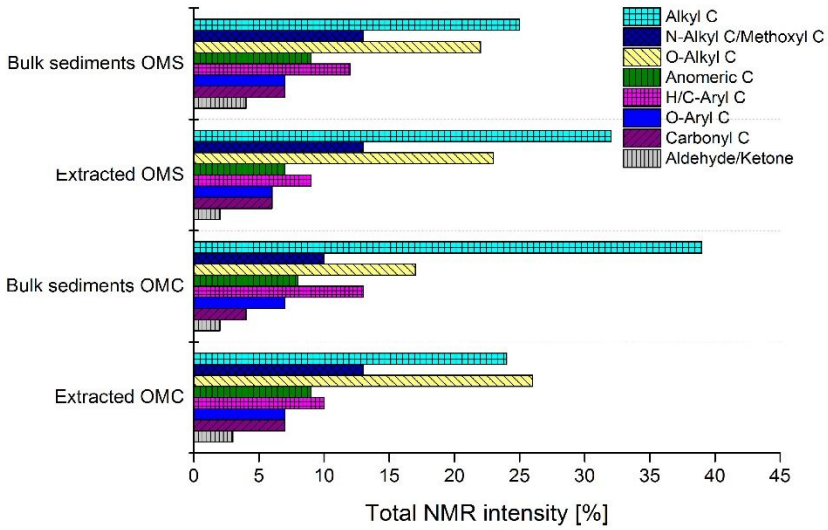
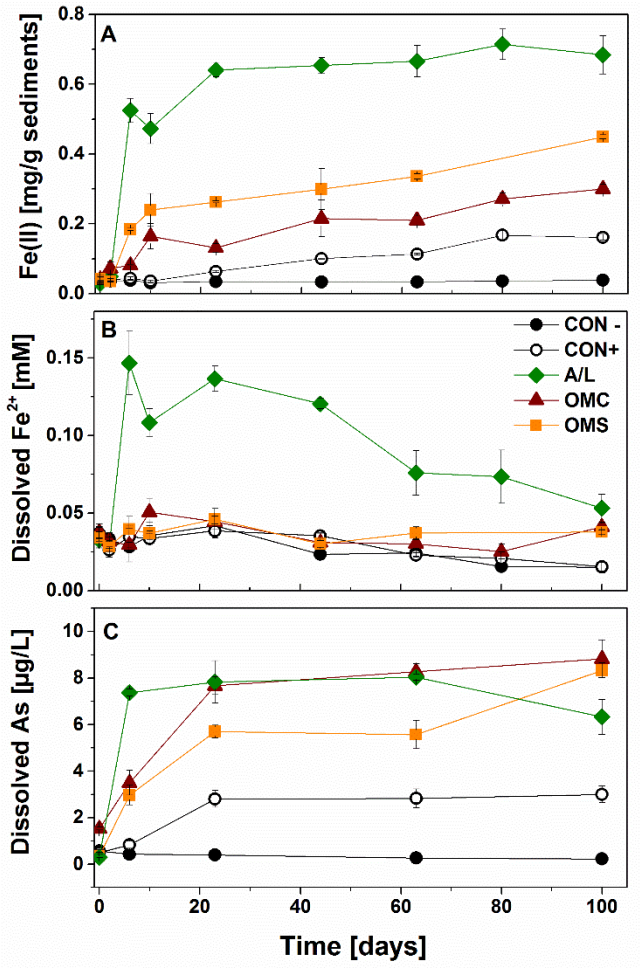


Figure 1. Characterization and comparison of the OM extracted from the Van Phuc aquitard (clayey silt) and aquifer (sandy) sediments, i.e. OMC and OMS. A) FTIR spectra with assigned peaks and potential C compounds: C-O, C-O-C, O-H (polysaccharides), COO- (amino/fatty acids), OH (cellulose), C=C, C=O (lignin-

767 derivatives), and B) shows the distribution of C-containing structural components
768 quantified by ^{13}C-NMR analysis.
769



770
771
772 Figure 2. Changes of Fe(II) in the sediment, dissolved Fe^{2+} and dissolved As over 100
773 days of incubation of As-bearing sediments in microcosms supplied with different C
774 sources. A) concentration of Fe(II) in the sediment quantified by 1 h digestion with 6 M
775 HCl, B) concentration of aqueous Fe^{2+}, C) dissolved As (please note that this is the As
776 mobilized from 1 g of sediments into 5 mL volume of artificial groundwater). Biotically
777 active control without additional C (CON+), abiotic control supplied with 160 mM
778 NaN_3 in order to inhibit microbial activity and amended with acetate/lactate (CON -),
779 and three microbially active setups amended with different C sources: OM extracted
780 from clayey silt sediments (OMC), OM extracted from sandy sediments (OMS),
781 acetate/lactate (A/L), at 12 mg C/L each. Error bars represent standard deviation from 3
782 vials. Each vial was measured in triplicate.

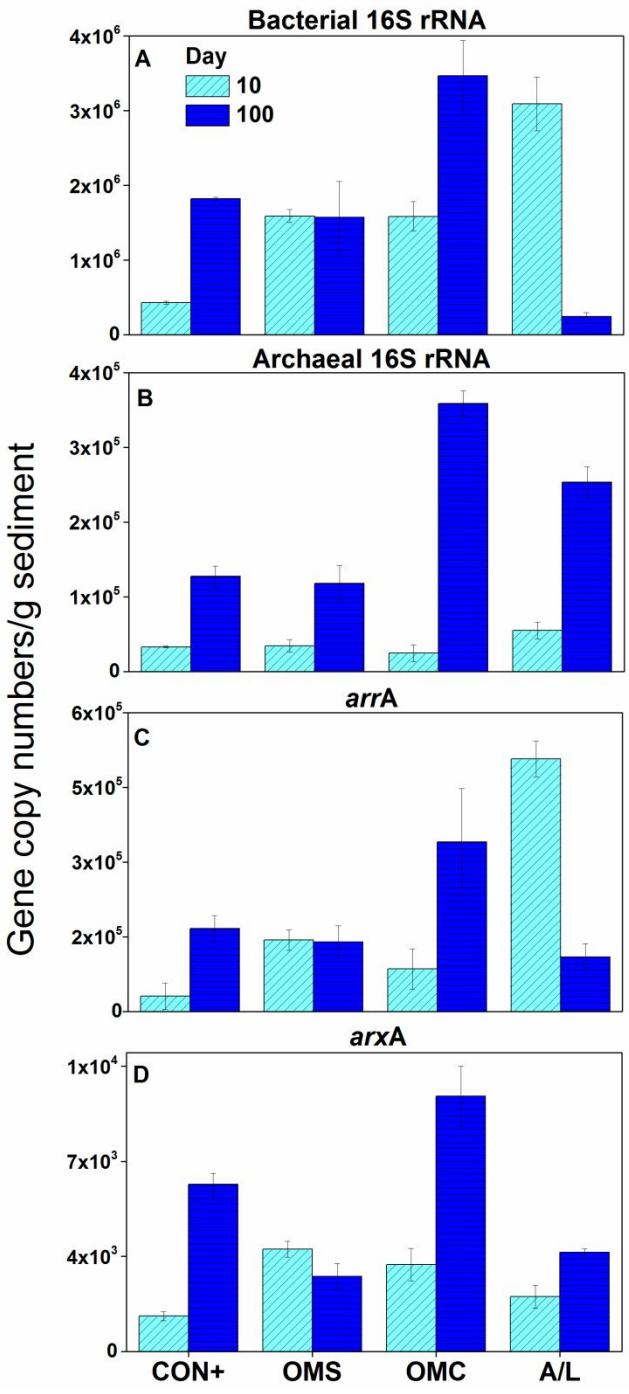


Figure 3. Quantitative PCR analysis of A) bacterial 16S rRNA gene, B) archaeal 16S rRNA gene, C) arsenate reductase gene (*arr*A) and D) anaerobic arsenite oxidase (*arx*A) gene copy numbers after 10 and 100 days of incubations with various C sources. Biotically active control without additional C (CON+), 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.

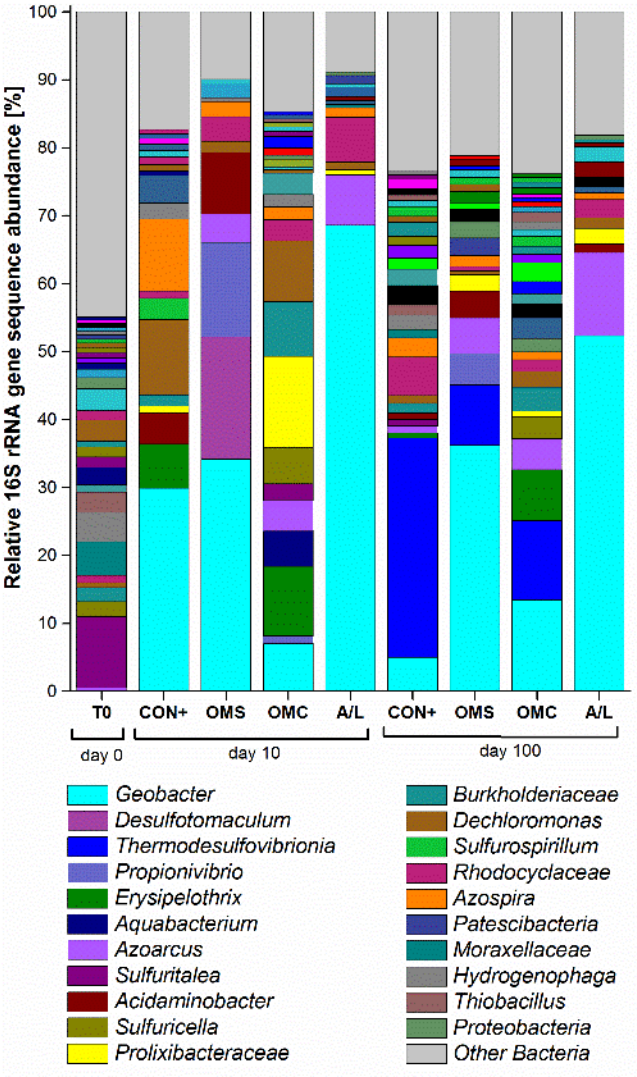


Figure 4. Changes in microbial community composition within 10 and 100 days of incubation with various C sources. The presented taxa were analyzed at genus level (and labelled with highest descriptive taxonomic level) and minimum abundance level of 0.5%. Biotically active control without additional C (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.