Conductive Particles Enable Syntrophic Acetate Oxidation between Geobacter and Methanosarcina from Coastal Sediments


ABSTRACT Coastal sediments are rich in conductive particles, possibly affecting microbial processes for which acetate is a central intermediate. In the methanogenic zone, acetate is consumed by methanogens and/or syntrophic acetate-oxidizing (SAO) consortia. SAO consortia live under extreme thermodynamic pressure, and their survival depends on successful partnership. Here, we demonstrate that conductive particles enable the partnership between SAO bacteria (i.e., Geobacter spp.) and methanogens (Methanosarcina spp.) from the coastal sediments of the Bothnian Bay of the Baltic Sea. Baltic methanogenic sediments were rich in conductive minerals, and had an apparent isotopic fractionation characteristic of CO2-reductive methanogenesis, and were inhabited by Geobacter and Methanosarcina. As long as conductive particles were delivered, Geobacter and Methanosarcina persisted, whereas exclusion of conductive particles led to the extinction of Geobacter. Baltic Geobacter did not establish a direct electric contact with Methanosarcina, necessitating conductive particles as electrical conduits. Within SAO consortia, Geobacter was an efficient [13C]acetate utilizer, accounting for 82% of the assimilation and 27% of the breakdown of acetate. Geobacter benefits from the association with the methanogen, because in the absence of an electron acceptor it can use Methanosarcina as a terminal electron sink. Consequently, inhibition of methanogenesis constrained the SAO activity of Geobacter as well. A potential benefit for Methanosarcina partnering with Geobacter is that together they competitively exclude acetoclastic methanogens like Methanothrix from an environment rich in conductive particles. Conductive particle-mediated SAO could explain the abundance of acetate oxidizers like Geobacter in the methanogenic zone of sediments where no electron acceptors other than CO2 are available.

IMPORTANT Acetate-oxidizing bacteria are known to thrive in mutualistic consortia in which H2 or formate is shuttled to a methane-producing Archaea partner. Here, we discovered that such bacteria could instead transfer electrons via conductive minerals. Mineral SAO (syntrophic acetate oxidation) could be a vital pathway for CO2-reductive methanogenesis in the environment, especially in sediments rich in conductive minerals. Mineral-facilitated SAO is therefore of potential importance for both iron and methane cycles in sediments and soils. Additionally, our observations imply that agricultural runoff or amendments with conductive chars could trigger a significant increase in methane emissions.

KEYWORDS Desulfuromonadales, Geobacter, Methanosarcina, nanoSIMS, activated carbon, competitive exclusion, direct interspecies electron transfer, syntrophic acetate oxidation

Received 28 January 2018 Accepted 28 March 2018 Published 1 May 2018 Citation Rotaru A-E, Calabrese F, Stryhanyuk H, Musat F, Shrestha PM, Weber HS, Snoeyenbos-West OLO, Hall POJ, Richnow HHR, Musat N, Thamdrup B 2018. Conductive particles enable syntrophic acetate oxidation between Geobacter and Methanosarcina from coastal sediments. mBio 9:e00226-18. https://doi.org/10.1128/mBio.00226-18. Editor Stephen J. Giovannoni, Oregon State University Copyright © 2018 Rotaru et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license. Address correspondence to Amelia-Elena Rotaru, arotaru@biology.sdu.dk, or Niculina Musat, niculina.musat@ufz.de.
Syntrophic acetate-oxidizing (SAO) bacteria live in a mutualistic interaction with methanogenic archaea, which feed on the $\text{H}_2$ or formate released by the SAO bacterial partner (1). Besides $\text{H}_2$ or formate, cysteine can also be used to transfer electrons in some SAO consortia (2). Several studies with synthetic consortia have shown SAO activity in members of the phyla Firmicutes (Thermacetogenium, Clostridium, Thermotoga, Candidatus Contubernalis, and Syntrophaceticus) and Proteobacteria (Desulfomicrobium and Geobacter) (2–14). Remarkably, acetoclastic methanogens (Methanosarcina and Methanothrix) have been proposed to play the role of syntrophic acetate oxidizers when provided with an appropriate $\text{H}_2$-consuming partner (15, 16). Some of the genera above have been suggested to carry out SAO in thermophilic digesters (17–26), lake/river sediments (21, 27, 28), tropical wetland soil (29), rice paddies (30–32), or oil field reservoirs (33). Many of these environments are rich in (semi)conductive minerals like magnetite (34, 35), pyrite (36, 37), or black carbon resulting from incomplete burning of plant biomass (38–40). Electrically conductive iron oxide minerals and carbon chars (magnetite, granular activated carbon, biochar) were previously shown to stimulate direct interspecies electron transfer (DIET), a recently described form of interspecies electron transfer (12, 41–49), whereas strict $\text{H}_2$-based interactions were shown to remain unaffected by the addition of conductive materials (44). DIET is a syntrophic association where electrons are transferred via conductive and/or redox-active cell surface structures between an electron-donating species (electron donor) and an electron-accepting species (electron acceptor) (47–49). Conductive minerals seem to alleviate the need for cells to produce certain cell surface molecules required for DIET (41). DIET mediated by conductive materials is considered a novel strategy to stimulate recalcitrant organic matter decomposition in anaerobic digesters (50–52) and to enhance methanogenic decomposition of organics in rice paddies (46, 53) and aquatic sediments (28, 54). It is likely that conductive materials replace the molecular conduits that cells require to establish direct contacts during DIET.

Although SAO via DIET is considered thermodynamically favorable at pH values between 1.9 and 2.9 and impossible at pH 7 (55), conductive minerals have been shown to facilitate SAO in synthetic denitrifying consortia at pH 7 (56). Nevertheless, the impact of minerals on environmentally relevant SAO is presently not understood. Mineral-facilitated SAO (here called mineral-SA0) could be significant in coastal environments rich in (semi)conductive minerals (36, 57–59). Such (semi)conductive minerals are likely to impact microbial processes (36, 56), for which acetate is a central intermediate (88–90).

Here, we investigated the role of mineral-SA0 in methanogenic processes from coastal sediments. We examined if electrically conductive materials mediate SA0 between Geobacter and Methanosarcina organisms coexisting in the brackish, iron-rich coastal sediments of Bothnian Bay. Our results indicate that mineral-SA0 may impact both the iron and the methane cycles in these sediments, with implications for atmospheric methane emissions.

RESULTS AND DISCUSSION

In this study, we found that methanogenic communities from Bothnian Bay made use of (semi)conductive particles to facilitate SAO. For this, we used a combination of physiological and stable isotope labeling experiments followed by monitoring of labeled products and incorporation of the labeled substrate in phylogenetically assigned cells by using nanoscale secondary ion mass spectrometry (nanoSIMS) coupled with catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH).

Syntrophic acetate oxidizers are difficult to enrich (57), because SAO is thermodynamically challenging (55). Here, we successfully enriched SAO consortia from temperate sediments (sediment temperature, 15°C; incubation temperature, 20 to 25°C) by successive cultivation in the presence of electrically conductive (>1,000 S/m [58]) granular activated carbon (GAC).

Characteristics of the Bothnian Bay methanogenic zone. (i) Geochemistry. Our hypothesis was that a high conductive mineral content would stimulate electric...
interactions between abundant electroactive microorganisms coexisting in the methanogenic zone. The Bothnian Bay sediments are rich in conductive minerals dispersed either within the fine structure of sediments or within ferromanganese nodules (59).

To explore mineral-mediated interactions in Bothnian Bay, we sampled the methanogenic zone of these sediments to verify the mineral content. Sediment cores were collected from 15-m water depth at station RA2, located at 65°43.6′N and 22°26.8′E in Bothnian Bay (Fig. 1), which had high sediment temperature (15°C) and low in situ salinity (0.5). The mineral content was low in manganese oxides (13 ± 3 μmol/cm³ [mean ± standard deviation] from both HCl and dithionite extractions), high in FeS, FeCO₃, and other poorly crystalline Fe-minerals (229 ± 8 μmol/cm³), and high in crystalline iron oxides (dithionite-extractable iron, 131 ± 4 μmol/cm³) and conductive magnetite (32 ± 7 μmol/cm³ oxalate extractable). This estimate of the magnetite content was similar to what has been previously observed below the sulfate-methane transition zone in Baltic Sea sediments (ca. 30 μmol/cm³) (60).

Besides iron oxide minerals, previous studies showed that black carbon, also a conductive material (40), dominated the coastal sediments of the Baltic Sea, representing 1.7% to 46% of the total organic carbon (TOC) in sediments closer to coastal towns (61). Conductive materials could reach Bothnian Bay by river runoff from the eight rivers entering the bay from Sweden and Finland, and also via runoff from the forestry industry and various coastal industries (59, 62).

The high abundance of conductive particles likely stimulates electrical interactions between abundant electroactive microorganisms that coexist in the methanogenic zone (41–43, 45, 52). Methane reached its highest concentrations below 25 cm depth (Fig. 1). In the methanogenic zone, two independent processes, SAO and/or acetoclastic methanogenesis, could consume acetate, a key intermediate of organic matter decomposition. SAO bacteria would need a CO₂-reductive methanogenic partner to scavenge the electrons released during acetate oxidation. To find out if CO₂-reductive methanogenesis was occurring in these sediments, we looked at the apparent isotopic fractionation of dissolved organic carbon (DIC, which includes CO₂, carbonic acid, bicarbonate, and carbonate) and methane. Methane was strongly depleted in ¹³C relative to DIC.
(median δ^{13}CH_{4} −74‰, median δ^{13}DIC, −2.5‰) (Fig. 1), which resulted in a signature apparent isotopic fractionation (α_c) of 1.07, characteristic of CO_{2}-reductive methanogenesis (63).

(ii) Microbial community. DIET consortia (Geobacter and Methanosarcina) can usually form more efficient electron transfer associations via conductive minerals than they do in their absence (42–44, 64). In contrast, H_{2}-transferring consortia have been shown to remain little affected by conductive materials (44, 65). We predicted that Bothnian Bay sediments rich in conductive mineral content, CO_{2}-reductive methanogenesis prevailed, and (iii) Methanosarcina and
Electrogens cohabited, we anticipated that mineral DIET could occur in the methano-
genic zone of Bothnian Bay. We tested this hypothesis in sediment incubations with or
without the addition of exogenous conductive particles.

Conductive GAC facilitated methane production from acetate (Fig. 2) and other
substrates (ethanol, butyrate, and glucose) that were degraded via acetate (Fig. S3F).
Tests with conductive magnetite showed that it stimulated methanogenesis even more
than GAC (Fig. S4F). On the other hand, nonconductive glass beads did not facilitate
methanogenesis from ethanol (Fig. 3SF), as these mixtures produced as much methane
as incubation mixtures without GAC \(P = 0.45\). However, GAC was the preferred
conductive particle, because we could concentrate rigorously on electron transfer (42),
whereas with use of (semi)conductive magnetite \(Fe_2O_4\) its \(Fe^{III}\) content could
additionally drive iron reduction, especially during long-term incubations (67, 68).

**Syntrophic acetate oxidation mediated by GAC.** Repeated transfers of the SAO
cultures with acetate as electron donor, \(CO_2\) as electron acceptor, and GAC produced
methane much faster than GAC-free controls and led to sediment-free cultures en-
riched in Desulfuromonadales (Geobacter and Desulfuromonas) and Methanosarcina
(Fig. 2). The enriched Desulfuromonadales were related to acetate oxidizers like \(G.\ psy-
chrophilus\) with \(97\%\) sequence identity and \(D.\ michiganensis\) \(98\%\) sequence identity)
(Fig. 3). The only methanogens detected in mud-free enrichments were related to
Methanosarcina \(subterranea\) \(99\%\) sequence identity) (Fig. 3). In the absence of con-
ductive minerals, Geobacter and Methanosarcina became undetectable after several
mud-free transfers (Fig. 2), and a filamentous Archaea (a Methanothrix-like morphotype)
took over acetate-only incubation mixtures (Fig. 2; Fig. S5F).

In incubation mixtures with acetate and GAC, acetate could be consumed by
acetoclastic methanogens and/or SAO consortia. A schematic representation of SAO
mediated by GAC tied to methanogenesis is presented in Fig. 4. Our hypothesis was
that during SAO, Geobacter cells donate electrons from the oxidation of acetate to GAC,
which then plays the role of a transient electron acceptor. Then, \(Methanosarcina\) cells
retrieve the electrons from GAC in order to reduce \(CO_2\) to methane.

To distinguish between acetoclastic methanogenesis and SAO, cultures were incu-
bated with \(_{13}CH_3_{12}COOH\). If acetoclastic methanogens utilized the \([_{13}C]methyl on

---

**FIG 3** Maximum likelihood trees of Bacteria and Archaea enriched in a seventh mud-free transfer with acetate and GAC. (a) A maximum likelihood tree of representative bacterial sequences from a mud-free transfer with conductive particles (GAC), under conditions strictly promoting methanogenic respiration. Acetate-oxidizing Desulfuromonadales dominated the 16S rRNA clone library, with more than half displaying close relationships to Geobacter psychrophilus \((97\%\) identity) and the rest to Desulfuromonas michiganensis \((98\%\)). The only methanogens enriched on acetate and GAC were relatives of Methanosarcina \(subterranea\) \(99\%\) identity), as shown in the maximum likelihood tree in panel b.
acetate, they would only produce $^{13}$CH$_4$. However, if SAO bacteria utilized $[13C]$acetate, then they would produce $^{13}$CO$_2$ (Fig. 4). When acetoclastic methanogens and SAO bacteria use $[13C]$methyl on acetate at the same time, both $^{13}$CO$_2$ and $^{13}$CH$_4$ would be produced. Our results support the latter model.

(i) SAO dependency on GAC. Incubations for ca. 70 days with $[13C]$acetate and GAC converted the $[13C]$methyl on acetate to $^{13}$CO$_2$, whereas control cultures lacking GAC produced little $^{13}$CO$_2$ (Fig. 4). This indicated that indeed GAC stimulated SAO.

(ii) Respiratory metabolism and SAO. During exponential growth (day 21), SAO could explain 27% of the total respiratory metabolism, whereas 27.4% could be explained by acetoclastic methanogenesis (Fig. 4). During stationary phase (day 63), SAO justified 8.4% of the total respiratory metabolism, whereas acetoclastic methanogenesis justified 61.8%.

(iii) Biosynthetic metabolism and SAO. The increase in abundance of Geobacter cells over time (Fig. 2) in incubation mixtures with GAC indicated that they could play the role of syntrophic acetate oxidizers in mineral-mediated SAO syntrophy. This was confirmed by analysis of the $^{13}$CH$_3^12$COOH-incubated SAO consortia by using nanoSIMS/CARD-FISH, an approach that helps correlate phylogeny and function (78). During incubation with GAC, both Geobacter and Methanosarcina cells became greatly

---

FIG 4 Experimental approach and evidence for SAO. (a) Experimental approach to distinguish between SAO and acetoclastic methanogenesis based on isotopic labeling. $^{13}$CH$_3^12$COOH was provided as 10% of the total acetate, which played the role of the electron donor for SAO consortia from the Bothnian Bay. During SAO, acetate-oxidizing Geobacter cells are expected to produce $^{13}$CO$_2$ ($^{13C}$, depicted in orange) and to incorporate $[13C]$acetate. During SAO, $^{13}$CO$_2$ will be diluted by the bicarbonate in the medium and should not generate significant $^{13}$CH$_4$. However, acetoclastic methanogenesis by Methanosarcina cells will generate $^{13}$CH$_4$ from $^{13}$CH$_3^12$COOH, while cells incorporate $[13C]$acetate in their cell mass. Cells expected to incorporate $[13C]$acetate are encircled in orange. (b) SAO activity was validated by using labeled $^{13}$CO$_2$ production from acetate, especially in SAO consortia provided with GAC (blue) versus cultures without GAC (orange). (c) An overview of acetate catabolism and how much is used for respiration by Geobacter versus acetoclastic methanogenesis by Methanosarcina.

---

<table>
<thead>
<tr>
<th>$^{13}$C-label</th>
<th>Day 0**</th>
<th>Day 21**</th>
<th>Day 63***</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}$CH$_3^12$COOH acetate (mM)</td>
<td>980 ± 33</td>
<td>821 ± 66</td>
<td>181 ± 23</td>
</tr>
<tr>
<td>Consumed $^{13}$CH$_3^12$COOH acetate (mM)</td>
<td>-</td>
<td>159 ± 37</td>
<td>640 ± 35</td>
</tr>
<tr>
<td>$^{13}$CO$_2$ (mM detected in solution and equilibrated with headspace)</td>
<td>0 ± 0</td>
<td>43 ± 3</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>Estimated $^{13}$C-respiration (%) by SAO</td>
<td>NA</td>
<td>27.0%</td>
<td>8.4%</td>
</tr>
<tr>
<td>Estimated $^{13}$C-respiration (%) by acetoclastic Methanosarcina</td>
<td>NA</td>
<td>27.4%</td>
<td>61.8%</td>
</tr>
</tbody>
</table>

*10% of the acetate added was $^{13}$CH$_3^12$COOH, the rest is unlabeled acetate.
**Values are replicate of 4 independent sacrificial cultures
***Values are replicate of 2 independent sacrificial cultures
NA=not applicable
enriched in $^{13}$C, indicating label assimilation from acetate (Fig. 5a and b). During exponential phase (day 21), Geobacter cells were 6 times more abundant than Methanosarcina (Fig. 2). Therefore, the entire Geobacter population assimilated 5 times more acetate than the Methanosarcina population (Fig. 5). However, upon prolonged incubation (day 63), the number of Geobacter cells remained relatively constant, while Methanosarcina cells increased in abundance to match the Geobacter population (Fig. 2). As a consequence, during the late incubation phase, the Methanosarcina population assimilated 3-fold more acetate than Geobacter (Fig. 5).

The ratio of Geobacter to Methanosarcina cells in the original sediment (8:1) was more similar to that observed in incubation during exponential growth (6:1) than to that observed during stationary phase (1:1). During exponential growth, Geobacter cells incorporate a high amount of $^{13}$C label. Although nanoSIMS results indicated that Geobacter could be the primary acetate oxidizer in SAO consortia from the Baltic Sea (Fig. 5), Desulfuromonas might also play a significant role in the process.

(iv) SAO is coupled to methanogenesis via a conductive particle electron conduit. To verify if Methanosarcina was used as a terminal electron acceptor by the
 acetate oxidizers, we chemically inhibited the metabolic activity of the methanogen by using a methyl-coenzyme M analogue (10 μM 2-bromoethanesulfonate [BES]) (69). If the acetate oxidizers were able to respire GAC, independent of electron uptake by *Methanosarcina*, we should be able to decouple acetate utilization from methanogenesis. However, acetate utilization ceased as soon as methanogenesis was inhibited by BES (Fig. 6), indicating that the (exo)electrogenic syntrophic acetate oxidizer (*Geobacter*) used the *Methanosarcina* methanogen as an electron sink. *Geobacter*'s dependency on the methanogen could be explained either by an interspecies interaction mediated by GAC (42, 48, 64) or a direct association based on self-assembled molecular conduits on the surface of the cells (48, 49, 70). To resolve if cells adapted to carry a DIET type of interaction via redox-active surface conduits, we switched the highly enriched *Geobacter-Methanosarcina* consortia to a medium without conductive particles. Only *Methanosarcina* survived the change (Fig. 7; Fig. S5F), demonstrating that without a conductive surface, Baltic *Geobacter* could not forge connections with the methanogen on its own. This is in contrast with previous studies on synthetic *Geobacter-Methanosarcina* consortia (48, 64). *Geobacter*’s inability to establish an interspecies interaction with the methanogen in the absence of conductive particles suggests that *Geobacter* used the conductive particle as an electron conduit for extracellular electron transfer and *Methanosarcina* as an electron sink. In what way *Geobacter* releases electrons extracellularly onto GAC and in what way *Methanosarcina*, but not *Methanothrix*, retrieves electrons from GAC are yet unresolved. Nevertheless, the ability of *Methanosarcina* to interact with *Geobacter* via conductive particles would likely give this methanogen a competitive advantage over *Methanothrix* in mineral-rich environments like the Baltic Sea.

(v) **Exoenzymes and shuttles are not endogenously created.** Previous studies indicated that extracellular enzymes could act as manufacturers of diffusible chemicals (H₂, formate) which could be used for electron transfer to methanogens (71). To test this hypothesis, we spiked cultures with spent medium from a fully grown culture that
was filtered through a 0.2-μm filter. The spent medium should theoretically contain (exo)cellular enzymes or potential shuttles, and if these were involved in electron transfer between the microorganisms from the Bothnian Bay sediments we should see an increase in methanogenic rates. We did not notice an increase in methanogenic rates in spiked cultures compared to control cultures (Fig. S6F). This indicates that (exo)cellular enzymes/shuttles are unlikely to play a role in conductive particle-mediated SAO between Geobacter and Methanosarcina.

**Conclusion.** Here, we showed that syntrophic acetate oxidation was coupled to CO₂-reductive methanogenesis via conductive particles in mud-free Desulfuromonadales-Methanosarcina consortia from the Baltic Sea. Our results suggest that conductive particles are essential for syntrophic acetate oxidation coupled to CO₂-reductive methanogenesis in sediments. Mineral-SAO could have significant implications for the isotopic composition and the cycling of methane in aquatic sediments. Anthropogenic activity could enhance the input of conductive materials to sediments, ultimately increasing methane fluxes. Since methane is a powerful greenhouse gas, we must better understand such actuators of methane emissions in the environment.

**MATERIALS AND METHODS**

**Sampling and incubations.** During an expedition on board the RV Fyrbygarren in July 2014, we sampled sediment cores with a Gemini gravity corer. Three sediment cores were gathered at station RA2,
which is located near the Swedish shoreline (coordinates: 22°26.8' E, 65°43.8' N). Within 24 h after sampling, the sediment was partitioned into depth-profiled aliquots and fixed for biogeochemical and molecular analyses inside an on-deck N₂-inflatable glove bag, as described below in detail.

For incubations, we gathered methanogenic sediment from a depth of 30 to 36 cm and replaced the gas atmosphere with 2 × 10⁻⁶ Pa of N₂-CO₂ (80:20) mix. The 30-to-36-cm-depth sediment was stored at 4°C until we generated slurries with various substrates and minerals.

Slurries were prepared in the lab in an anaerobic chamber and were generated within 6 months after sampling. For slurries, we used 3-ml cut-off syringes to distribute 2.5 ml sediment into 20-ml gas-tight vials with 7.5 ml DSM 120-modified medium. The modified DSM 120 medium was prepared as described before (48) but with 0.6 g NaCl. Sediment slurries had a high organic content, whereas mud-free enrichments did not. Therefore, we amended the mud-free enrichments with 0.2 g/liter yeast extract from a 100-g/liter anaerobic and sterile stock, which is required for methanogenic growth. Before inoculation, the complete medium which lacked the substrate and (semi)conductive minerals was dispensed anaerobically by syringe into sterile degased vials with or without minerals prepared as described below.

Conductive materials, GAC (0.1 g/10 ml; Merck), and magnetite (0.1 g/10 ml; Sigma-Aldrich) were weighed, added to vials, overlaid with 200 µl ultrapure water for wet sterilization, degased for 3 min with an N₂-CO₂ (80:20) mix, and autoclaved at 121°C for 25 min. Control experiments were carried out with acid-washed glass beads instead of conductive minerals. Substrates (5 mM glucose, 5 mM butyrate, 10 mM acetate, 10 mM ethanol) were added to media from sterile anoxic 1 M stocks by asceptic and anaerobic techniques. Control experiments were carried out without additional substrate to learn if the organic compounds in sediment could be used as substrates for methanogenesis. All incubations were carried out at room temperature (20 to 23°C) in triplicate unless otherwise noted.

Gas samples were withdrawn at timed intervals using hypodermic needles connected to a syringe closed by an airtight valve. Gas samples (0.5 ml) were stored, until measured, by displacing 0.5 ml ultrapure water, which filled 3-ml Exetainers. Thirty-microliter gas samples were tested for methane on a Thermo Scientific gas chromatograph equipped with a TG-Bond Msizee 5A column (30 m by 0.53 mm by 50 µm) and a flame ionization detector (FID). The carrier was N₂ (flow rate, 5 ml/min), and we used an isothermal oven temperature of 150°C with the injector and detector set at 200°C. Gas standards (0.01%-50% CH₄ in N₂) from Mikrolab Aarhus A/S were always run along with samples. Short-chain volatile fatty acids (SCVFA) were detected via high-performance liquid chromatography (HPLC) of 0.45-µm-filtered and 3-times-diluted samples. For HPLC, we used an Agilent 1100 instrument equipped with an Aminex-HPX 87H column heated at 70°C and a VWR detector, which detects SCVFA at 210 nm. Five millimoles of sulfuric acid was used as eluent at a flow rate of 0.6 ml/min. Standards used ranged between 0.1 mM and 10 mM. The detection limit for all SCVFA was 100 µM.

**Biogeochemical analyses.** To determine biogeochemical parameters, we took sediment aliquots from every 2 cm in an anaerobic glove bag filled with N₂ gas. At this station, the sulfide-methane transition zone was below 15 cm. Geochemical parameters of direct relevance to this work were methane, dissolved inorganic carbon (DIC), and resident iron and manganese oxide species. For in situ methanogenesis, isotopic fractionation and ¹³C/¹²C-methane isotopic fractionation, we blocked the activity of the microorganisms by immersing 2 ml active sediment into 4 ml of 2.5% NaOH. NaOH-treated samples kept for 20°C until extraction of the different Fe-oxides except for magnetite), and oxalate extraction (to dissolve magnetite) (68, 73), followed by a ferrozine assay (74). For analysis of manganese, extractions were carried out as described for solid iron, and concentrations in the supernatant were analyzed undiluted by flame atomic absorption spectroscopy.

For pore water parameters, porosity of the sediments was calculated from the relationship between the wet weight of the sediment and its dry weight. For pore water extraction, 50 ml sediment was sampled every 2 cm by scooping sediment into Falcon tubes, from which pore water was extracted with the use of rhizons (rhizosphere; pore size, 0.2 µm). Pore water work was carried out under a N₂ atmosphere in a glove bag.

For pore water Fe²⁺ and Mn²⁺ concentrations, 1 ml pore water was mixed with 20 µl 6 N HCl and stored at −20°C. Soluble Fe²⁺ in the pore water was determined using the ferrozine assay (74).

Pore water DIC was sampled inside an N₂-filled glove bag on board. DIC samples were filled to brim to ensure no gas bubbles into 3-ml glass vials, which contained 20 µl HgCl₂-saturated water. Samples were stored upside down at 4°C until measurements. For measurements, we converted DIC to CO₂ by acidification with 50 µl undiluted H₃PO₄ for each 200-µl DIC sample. CO₂ was allowed to equilibrate in the headspace overnight inside 12-ml He-flushed Exetainers. DIC concentration and the [¹³C/¹²C]DIC isotope ratios were measured on an isotope ratio mass spectrometer coupled to a gas bench, as previously described (75).
**Molecular analyses.** For molecular analyses, we sampled 2 ml from every 2 cm of sediment depth. Samples were collected using cut-off syringes at the same time with samples for biogeochemical parameters, on board and inside an anaerobic bag. For safe storage during transportation, 3 depths, so a total of 6 cm, were pooled together and mixed with 6 ml MoBio RNAlater (1:1). Prior to DNA extractions, RNAlater was removed by centrifugation. For DNA extraction, we used the MoBio RNA soil kit coupled to a cDNA soil kit and followed the instructions provided by the kit manufacturer. DNA was quantified using a Nano Drop before downstream applications.

**Quantitative PCR.** To target electrogenic microorganisms, genus/order-specific PCR was performed with primers for Desulfuromonadales (includes all Geobacter), Geothrix, Rhodoferrax and Shewanella. For methanogens, the following genus/order-specific primers were tested to target: Methanosarcinaceae, Methanothrix, Methanococcales, Methanobacteriales, Methanomicrobiales. A list of all the primers used, making of standards, and the conditions for quantitative PCR (qPCR) are available in Table S1F and Text S1, respectively.

**16S rRNA gene sequencing, library preparation, and phylogenetic tree reconstruction.** 16S rRNA gene MiSeq amplicon sequencing was carried out from the 30-to-36-cm-depth interval of triplicate cores. Details on the procedure can be found in Text S1. Qualitative and quantitative information regarding MiSeq sequence reads can be found in Fig. S1 in the supplemental material. Amplification of partial Geobacter and Methanosarcina 16S rRNA gene sequences was done as described before (76). Cloning employed the TOPO TA cloning kit (Thermo, Fisher Scientific) followed by direct sequencing of PCR products from cloned plasmid DNA (Macrogen). Maximum likelihood phylogenetic trees were constructed using Geneious (77).

**13C labeling experiments.** Cultures were incubated with a 1:9 mix of $^{13}$CH$_3$COOH and unlabeled acetate. Approximately 21 cultures with GAC and 16 for the GAC-free cultures were started for the nanoSIMS experiment, because we would sacrificially harvest three at each time point. Headspace gas samples and VFA samples were analyzed as above.

We followed enrichment of $^{13}$CO$_2$ over time by using IR-MS. Briefly, 2.5-ml media samples were retrieved anaerobically for $^{13}$CO$_2$ analyses and immediately stored with 20 µl HgCl$_2$-saturated water, without any headspace, and acidified as explained above for DIC analyses in sediment samples; finally, IR-MS analyses were carried out manually against CO$_2$ gas standards and bicarbonate standards.

We followed the incorporation of labeled acetate ($^{13}$CH$_3$COOH) into a specific phytype by using CARD-FISH coupled to nanoSIMS, as described below (78).

**CARD-FISH.** To count cells of a specific phylogenetic group and label cells prior to nanoSIMS, we used CARD-FISH as described previously (79) and the following probes: Non338 (80) to check for nonspecific binding, Eub338I-III (81,82) to target Eubacteria, Geo3a-c in equimolar amounts with helpers H-Geo3-3 and H-Geo3-4 to target the Geobacterales cluster (83); Arch915 (72) to target Archaea, and MS821 (72) to target Methanosarcina species. A detailed description of the CARD-FISH protocol can be found in Text S1.

**Quantitative imaging of 13C label incorporation via nanoSIMS.** Chemical imaging and quantitative analysis of $^{13}$C label incorporation was carried out on a NanoSIMS-50L instrument (Cameca, Ametek) operating in negative extraction mode. nanoSIMS analyses were carried out on laser microdissection-selected fields, and the collected data were quantitatively analyzed using the LANS software (84). A detailed description of the protocol used for nanoSIMS analyses and data collection can be found in Text S1.

**Accession number(s).** Sequence files for our partial Geobacter and Methanosarcina 16S rRNA gene sequences and 16S MiSeq sequence data can be found at NCBI under BioProject ID PRJNA415800.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00226-18.

**TEXT S1,** DOX file, 0.2 MB.
**FIG S1,** PDF file, 0.2 MB.
**FIG S2,** PDF file, 0.04 MB.
**FIG S3,** PDF file, 0.05 MB.
**FIG S4,** PDF file, 0.03 MB.
**FIG S5,** PDF file, 1.2 MB.
**FIG S6,** PDF file, 0.03 MB.
**TABLE S1,** DOCX file, 0.1 MB.
**TABLE S2,** DOCX file, 0.05 MB.

**ACKNOWLEDGMENTS**

This work is a contribution to Danish Research Council (DFF) grant 1325-00022 to A.-E.R. DFF grant 4181-00203 supported O.S.W. The RV Frybyggaren expedition was cofinanced by a Swedish Research Council (VR) grant to Phys A DFF (grant 4002-00521) to B.T.

We acknowledge the Centre for Chemical Microscopy (ProVIS) at the Helmholtz...
REFERENCES


