Inhibition of sulfate-reducing bacteria with formate

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One sentence summary: Formate treatment can delay and inhibit growth rate and sulfate reduction of sulfate-reducing organisms species-specific.

Abstract

Despite hostile environmental conditions, microbial communities have been found in μL-sized water droplets enclosed in heavy oil of the Pitch Lake, Trinidad. Some droplets showed high sulfate concentrations and surprisingly low relative abundances of sulfate-reducing bacteria in a previous study. Hence, we investigated here whether sulfate reduction might be inhibited naturally. Ion chromatography revealed very high formate concentrations around 2.37 mM in 21 out of 43 examined droplets. Since these concentrations were unexpectedly high, we performed growth experiments with the three sulfate-reducing type strains *Desulfovibrio vulgaris*, *Desulfobacter curvatus*, and *Desulfococcus multivorans,* and tested the effects of 2.5, 8, or 10 mM formate on sulfate reduction. Experiments demonstrated that 8 or 10 mM formate slowed down the growth rate of *D. vulgaris* and *D. curvatus* and the sulfate reduction rate of *D. curvatus* and *D. multivorans*. Increasing formate concentrations delayed the onsets of growth and sulfate reduction of *D. multivorans*, which were even inhibited completely while formate was added constantly. Contrary to previous studies, *D. multivorans* was the only organism capable of formate consumption. Our study suggests that formate accumulates in the natural environment of the water droplets dispersed in oil and that such levels are very likely inhibiting sulfate-reducing microorganisms.

Introduction

Sulfate-reducing bacteria constitute a prevalent problem in gas and oil production because they generate hydrogen sulfide which causes corrosion of pipelines or equipment and lowers oil quality. Resulting environmental hazards and massive financial losses demand strategies to inhibit the activity of sulfate-reducing bacteria (Popoola *et al.* 2013, Chen *et al.* 2021). Conventional methods for the inhibition of sulfate-reducing microorganisms such as antibiotics or formaldehyde and glutaraldehyde are expensive and often problematic for the environment (Rossmoore and Sondossi 1988, Sondossi, Rossmoore and Williams 2001, de Jesus *et al.* 2015, Xue and Voordouw 2015, Liu *et al.* 2018, Di Martino 2021). Hence, alternative strategies have been developed, such as the injection of nitrate and nitrate-reducing bacteria to oxidize hydrogen sulfide or to outcompete sulfate-reducing organisms *in situ* (Halim, Gubner and Watkin 2011, da Silva *et al.* 2014, Zhang *et al.* 2021). Formaldehyde and glutaraldehyde are sometimes injected as a generic biocide, which is also used in other industries (Rossmoore and Sondossi 1988, Sondossi, Rossmoore and Williams 2001, Xue and Voordouw 2015, Di Martino 2021). Alternatively, sulfate reduction can be inhibited specifically by treatment with chemical analogues of sulfate, adenosine-5'-phosphosulfate (APS), and sulfite such as molybdate, selenate, and perchlorate (Stoeva and Coates 2019).

Besides these targeted approaches, the oil industry applies formate during drilling as environmentally friendly, biodegradable, and non-hazardous to human health-chemical to prevent corro-

sion and to maximize hydrocarbon yields (Downs 1993, Bungert *et al.* 2000, Sinomine Specialty Fluids 2020). Although commonly applied, the effects of formate on sulfate-reducing bacteria are so far unknown. Recently, however, we have made observations suggesting that formate may inhibit sulfate-reducing bacteria. In a series of studies, we investigated natural, oil-degrading microbial communities that live in tiny water droplets enclosed in the heavy oil of the Pitch Lake on Trinidad, the world's largest natural heavy oil seep (Meckenstock *et al.* 2014, Pannekens *et al.* 2020, Voskuhl *et al.* 2021). In these water droplets, only few sulfate-reducing bacteria were present despite high sulfate concentrations around 2.3 ± 0.99 mM (mean $\pm$ standard deviation; Meckenstock *et al.* 2014, Pannekens *et al.* 2020, Voskuhl *et al.* 2021). Analysis of community composition by 16S rRNA gene sequencing revealed that less than 0.2% of the operational taxonomic units (OTUs) could be assigned to known sulfate-reducing bacteria and archaea (Voskuhl *et al.* 2021), such as *Desulfobacteraceae*, *Desulfovibrionaceae*, *Desulfurococcaceae*, *Desulfuromonadaceae*, *Nitrospiraceae*, *Syntrophaceae*, *Syntrophobacteraceae*, and *Syntrophorhabdaceae* (Muyzer and Stams 2008). Normally, sulfate concentrations > 50 μM are expected to select for sulfate-reducing microorganisms over methanogens (Sierra-Garcia and de Oliveir 2013, Pannekens *et al.* 2019), but methanogenesis has been described as relevant process for hydrocarbon degradation and the formation of heavy oils over geological timescales (Gieg, Duncan and Suflita 2008, Jones *et al.* 2008). In the Pitch Lake, methanogenesis rather than sulfate reduction seems to be the terminal electron-

accepting process based on determined metabolites and the relative abundances of methanogenic archaea and marker genes associated with methanogenesis (Schulze-Makuch *et al.* 2011, Meckenstock *et al.* 2014). Interestingly, however, sulfate-reducing microorganisms from the Pitch Lake were successfully enriched in an experiment where Pitch Lake oil containing the indigenous microbiota was incubated anaerobically together with artificial salt medium that was designed to mimic the natural water chemistry (Pannekens *et al.* 2021). The enriched microorganisms reduced on average 8.5 mM sulfate within 2.5 years (Pannekens *et al.* 2021) suggesting that microbial communities from the Pitch Lake have the potential for sulfate reduction but might be naturally inhibited by oil reservoir chemicals or microbial metabolites in the water droplets.

While analysing ion compositions to characterize the geochemical conditions of individual water droplets (Voskuhl *et al.* 2021), we noticed exceptionally high formate concentrations never reported before for comparable environmental samples. Hence, we hypothesized that formate might be a possible inhibitor of sulfate-reduction in the Pitch Lake. This hypothesis was reinforced by a study, which demonstrated that high formate concentrations accumulated in *Escherichia coli* cultures during mixed acid fermentation and that concentrations of 20 mM formate delayed the growth of *E. coli* by acidic stress, causing decoupling of the membrane potential (Kirkpatrick *et al.* 2001). Formate is a common fermentation product (Knappe and Sawers 1990, Crable *et al.* 2011) and may accumulate in the surroundings of cells, provoking inhibitory effects at high concentrations. However, formate may not only have negative effects, but it may also serve as a growth substrate that can be oxidized to CO_2 by the periplasmic enzyme formate dehydrogenase (FDH). The gained electron equivalents can be used for respiration. Formate can also be used for carbon fixation *via,* e.g. the Wood–Ljungdahl pathway (WLP; Crable *et al.* 2011).

In this study, we investigated whether formate may inhibit sulfate reduction. In a first step, we measured formate concentrations in 43 water droplets from the Pitch Lake. We also determined the *in situ* availability of molybdenum, nickel, and selenium, because these elements can affect formate metabolism and sulfate reduction negatively. A lack of trace element supply of molybdenum, nickel, and selenium can be limiting for FDH (Soini, Ukkonen, Neubauer 2008, McDowall *et al.* 2014), while high concentrations of molybdate and selenate are well-known inhibitors of sulfate reduction (Stoeva and Coates 2019). In a second step, generic inhibition by formate was studied by treating three sulfate-reducing bacterial strains with formate concentrations between 2.5 mM, as measured *in situ*, and 10 mM. To evaluate the effects of formate across a wide metabolic range, we selected the type strains *Desulfovibrio vulgaris,* performing incomplete lactate oxidation (Heidelberg *et al.* 2004, Pereira *et al.* 2008), *Desulfobacter curvatus*, performing acetate degradation *via* the citric acid cycle (TCA; Ekstrom, Morel and Benoit 2003), and *Desulfococcus multivorans,* performing acetyl-CoA degradation *via* the WLP; Ekstrom, Morel, Benoit 2003, Dörries *et al.* 2016, Liu *et al.* 2018).

Materials and methods
Sampling of Pitch Lake water droplets

The Pitch Lake in La Brea, Trinidad and Tobago, is the world's largest, natural asphalt lake. It represents an extraordinary microbial habitat, which has been described in detail by Voskuhl *et al.* (2021). Despite being called a 'lake', it does not consist of

water but of approximately 10 million tons of heavy oil covering an area of 47 hectares with a depth of $\sim$ 87 m. The asphalt lake is constantly nourished by heavy oil originating from a reservoir at a depth of approximately 1400 m (Attwooll and Broome 1954, Chilingarian and Yen 1979, Ostapkowicz *et al.* 2017). The heavy oil is hostile to life, but active microbial communities do thrive in tiny water droplets that occur enclosed in the oil (Meckenstock *et al.* 2014, Pannekens *et al.* 2020). Analyses of stables isotopes have proven that the water droplets originate from the deep oil reservoir, implying that the microbial communities therein originate from the deep reservoir as well (Meckenstock *et al.* 2014). In order to isolate the water droplets from the oil, we first sampled oil from the Pitch Lake surface, transferred it into sterile, gas tight glass jars, which were flushed with nitrogen and shipped to the lab, where they were stored at 4°C until use. To sample the water droplets, the oil-containing jars were heated to 45°C (resembling the *in situ* temperature) for 30 min. By that, the oil liquefied and water droplets ascended to the surface where they were picked with a 10 μL pipette (Pannekens *et al.* 2020, Voskuhl *et al.* 2021). Droplets were frozen at −70°C until used for ion chromatography.

Microorganisms and cultivation conditions

Three sulfate-reducing bacteria from the phylum Proteobacteria, order *Desulfobacterota* (formerly Deltaproteobacteria; Waite *et al.* 2020), namely *Desulfovibrio vulgaris* (DSM 644)*, Desulfobacter curvatus* (DSM 3379), and *Desulfococcus multivorans* (DSM 2059) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The three type-strains were chosen because of their different energy metabolism, namely incomplete lactate oxidation (*D. vulgaris*), TCA (*D. curvatus*), and WLP (*D. multivorans*). Additionally, the selection of the model organisms matches the microorganisms detected in the Pitch Lake water droplets since the genus *Desulfovibrio* and several members of the family *Desulfobacteraceae*, which includes the strains *D. curvatus* and *D. multivorans,* have been identified in the water droplets of the Pitch Lake (Pannekens *et al.* 2020).

D. vulgaris was grown with medium DSM 63 at 37°C without shaking, *D. multivorans* with medium DSM 195 at 35°C, and *D. curvatus* with medium DSM 193 at 30°C. *D. multivorans* and *D. curvatus* were incubated on a shaker at 150 rounds per minute (rpm) to avoid flocculation of the cells. Cultures were incubated under anoxic conditions in 200 mL serum flasks filled with 100 mL medium and closed with butyl stoppers. Cultures of *D. curvatus* and *D. multivorans* were flushed with a gas mixture of 80% N_2 and 20% CO_2 (BIOGON® C20, Linde, Germany), while cultures of *D. vulgaris* were flushed with pure nitrogen. The medium for *D. curvatus* and *D. multivorans* contained 20 mM sulfate and 15 mM for *D. vulgaris*. *D. vulgaris* was grown with 18 mM Na-DL-lactate, *D. curvatus* with 18 mM Na-acetate, and *D. multivorans* with 4.2 mM Na-benzoate as carbon and electron source.

Experiments with formate as inhibitor

Pre-cultures of *D. vulgaris*, *D. curvatus*, and *D. multivorans* were sampled in the stationary phase and inoculated 1:10 into freshly prepared medium DSM 63, DSM 193, and DSM 195, respectively. Initial cell numbers were about 1×10^5 mL^{-1} for *D. curvatus* and *D. multivorans* and about 1×10^7 mL^{-1} for *D. vulgaris,* because the latter turned out to be sensitive to high dilutions in preliminary experiments. The three organisms were grown under the following treatment conditions: (I) 0 mM formate, positive control; (II) 2.5 mM formate; (III) 8 mM (*D. vulgaris* and *D. curvatus*), *D. multivorans* was unintentionally cultivated with 10 mM formate; (IV)

0.08 mM molybdate as inhibitor of sulfate reduction (inhibition control I; de Jesus *et al.* 2015); (V) 50 μM chloroform as inhibitor of the WLP (inhibition control II; Ekstrom, Morel, Benoit 2003); (VI) negative control, medium without electron-donor (*D. curvatus* and *D. multivorans*), or without sodium-thioglycolate and ascorbic acid (*D. vulgaris*); and (VII) sterile control, autoclaved directly after inoculation. *Desulfococcus multivorans* was also grown with (VIII) 10 mM formate, whereby 2.2 mM formate $\pm$ 1.13 were added daily from a 500 mM sodium-formate stock solution until a final formate concentration of 31 mM was reached. The daily formate addition in this treatment started at day 2 and lasted until day 12. For simplicity, this treatment is referred in the following as 31 mM formate treatment. Inhibitors in treatments (I)–(VI) and (VIII) were added 1 day after inoculation to allow for acclimatization. Sterile controls were singlets, all other treatments were performed in triplicates.

To avoid changes in concentrations, the added volume of the inhibitors formate, molybdate, and chloroform was adjusted to a volume of 500 μL. For compensating concentration discrepancies in comparison to untreated cultures, identical volumes of sterile and anoxic ultrapure water (18.2 MΩcm, TOC < 5 ppb; Millipore, Germany) were added where needed.

Sulfate and formate measurements via ion chromatography

To precipitate confounding medium compounds, 90 μL of sample were diluted in 250 μL 0.1 M KOH, incubated for approximately 15 min at room temperature, and stored at $-70°$C until further analysis. Subsequently, samples were thawed at room temperature, amended with 630 μL ultra-pure water and centrifuged at 18 000 relative centrifugal force (rcf) at 4$°$C for 5 min in an Eppendorf centrifuge. A total of 100 μL of the resulting supernatant were diluted 1:10 with ultra-pure water, resulting in a 1:500 dilution of the original sample. Sulfate and formate concentrations were separated and determined with a Dionex aquion ion chromatography system (Thermo Scientific, MA), equipped with a C_{18} guard column (Dionex Ion Pac™ AG23-4 μm RFIC™ 2 × 50 mm, Thermo Scientific), and analytical column (Dionex Ion Pac™ AS23-4 μm RFIC™ 2 × 250 mm, Thermo Scientific) running with the eluent 0.8 mM $NaHCO_3$ and 4.5 mM Na_2CO_3 at a flow rate of 0.25 mL min^{-1}. The columns were preserved at 30$°$C in a DionexUltiMate 3000 RS column compartment (Thermo Scientific). Chromeleon Software (Version 7.2 SR5, Thermo Scientific) was applied for peak analysis. The detection limit was 1 mM for sulfate and 0.6 mM for formate. In the exponentially growing cultures, the maximum rate of specific sulfate or formate oxidation was determined as the slope of the linear regression of sulfate or formate concentration plotted versus time. The linear section of each curve that was used for regression analysis had to be determined individually based on visual inspections of the graphs.

Formate and trace element analyses in Pitch Lake water droplets

A total of 2 μL of each water droplet were diluted 1:300 for ion chromatography measurements of sulfate and formate as described above. To assess a possible limitation of FDH activity by a possible natural lack of molybdenum, selenium, or nickel we determined their concentrations in 55 μL of pooled Pitch Lake water droplets and in the growth medium DSM193 utilized for *D. multivorans via* inductively coupled plasma mass spectrometry (ICP-MS). The trace element analyses were carried out with a quadrupole ICP-MS system (Perkin Elmer Sciex Elan DRC-e) operating at 1000 W plasma power, 14 L min^{-1} plasma gas flow, and 0.95 L min^{-1} nebulizer gas flow and an auto sampler system (Perkin Elmer AS-90) connected with a peristaltic pump with a sample flow of 1 mL min^{-1}. To avoid contamination and memory effects, the wash time between measurements was set to 10 s (with 1% HNO_3, suprapure). Before analyses, samples were diluted 1:2 (DSM193) and 1:200 (Pitch Lake water droplet pool) with 1% HNO_3 (suprapure) containing 10 ng L^{-1} of yttrium (Y) as internal standard.

Cell counting via flow cytometry

Samples for cell counts were taken every 2 h (*D. vulgaris*) or daily (*D. curvatus*, *D. multivorans*) from treatments (I)–(VII) until the stationary phase of the positive control (treatment I) was reached after 28 h (*D. vulgaris*), 9 days (*D. curvatus*), and 15 days (*D. multivorans*). A total of 1 mL of sample was removed from each culture in a sterile and anoxic way and an aliquot of 487 μL was immediately fixed in 1% formaldehyde (end concentration), incubated for 15 min at room temperature and frozen at $-70°$C until further use. For flow cytometric analysis, samples were thawed at room temperature, vortexed, and treated 1 min in an ultrasound bath to dissipate aggregated cells (S 0 H Elmasonic, Elma Schmidbauer GmbH, Germany). Then, samples were centrifuged for 5 s (centrifuge 5415D, Eppendorf, Germany) to remove precipitated minerals. *D. curvatus* and *D. multivorans* cells were analysed without staining, *D. vulgaris* was stained with 5 μM Syto9 (end concentration). Cells were enumerated with a flow cytometer (NovoCyte® Flow Cytometer, equipped with a FITC laser (488 nm), ACEA Biosciences, Inc.) either by analysing a fixed sample volume of 25 μL or by counting 50 000 items. Cells counts were based on light side-scattering and in the case of *D. vulgaris* also on green fluorescence of the counted particles with the threshold for the light side-scatter set to 1000 for *D. vulgaris* and *D. curvatus* and to 10 000 for *D. multivorans*. Specific growth rate was calculated as the slope of the regression line of the natural logarithm of cell density versus time.

Statistical analysis for experimental data

We used one-way analysis of variance (ANOVA) to test the effect of treatment on five different variables, i.e. (A) sulfate reduction rate (mM day^{-1}), (B) onset of sulfate reduction (day), (C) growth rates (day^{-1}), (D) onset of growth (day), and (E) total reduced sulfate (mM). Because the experimental design was not fully factorial, separate one-way ANOVAs were performed for each type strain. For *D. multivorans* we also used a one-way ANOVA to test the effect of treatment on (F) the formate oxidation rate (mM day^{-1}). A significant ANOVA result was followed up by a Tukey *post-hoc* test in order to identify the exact treatments, which differed significantly from the positive control (0 mM formate). All adjusted *P*-values from the Tukey test refer to a pairwise comparison with the positive control (0 mM formate). All statistical analyses were performed in R version 4.0.3 using R packages vegan and pairwiseAdonis (Martinez Arbizu 2020, Oksanen *et al.* 2020).

Results

Formate and trace element concentrations in the Pitch Lake water droplets

Formate concentrations were analysed in 43 water droplets to evaluate possible inhibitory effects *in situ*. Because the water droplets from the Pitch Lake oil were only a few μL in size (median 3 μL) and had to be diluted 1:300 for measurements, the detection limit for formate was relatively high (0.6 mM). In 21 out of 43 water
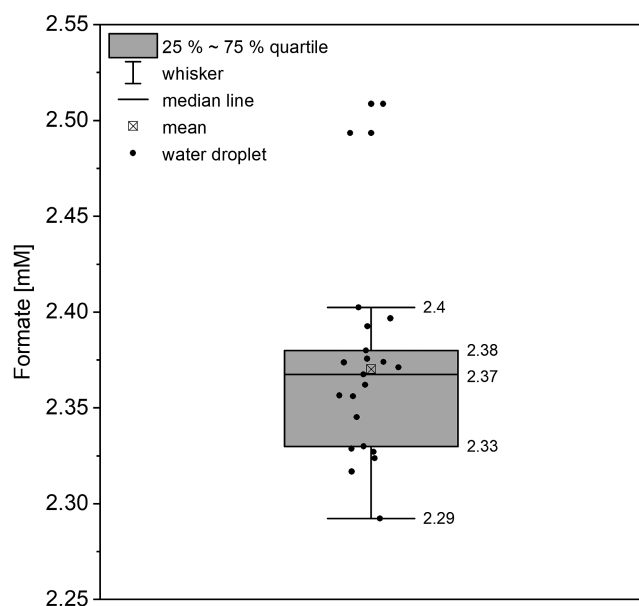
Figure 1. Formate concentrations in 21 individual water droplets isolated from heavy oil of the Pitch Lake, Trinidad.

droplets, formate concentrations were above the detection limit and showed a mean of 2.37 mM formate $\pm$ 0.05 mM (Fig. 1). To investigate if a potential lack of molybdenum, nickel, or selenium may cause the accumulation of formate in the water droplets by limiting the activity of the formate hydrogen lyase complex (Soini, Ukkonen, Neubauer 2008, McDowall *et al.* 2014), we compared the trace element concentrations in pooled Pitch Lake water droplets against the concentrations in the growth medium DSM 193 of *D. multivorans*. The pooled water droplets contained 16 μg L^{-1} molybdenum, 205 μg L^{-1} nickel, and 130 μg L^{-1} selenium, whereas the growth medium of *D. multivorans* contained only 13 μg L^{-1} molybdenum, 25 μg L^{-1} nickel, and selenium concentrations below the detection limit of 6.5 μg L^{-1}. As we did not observe formate accumulation in the experimental cultures of *D. vulgaris*, *D. curvatus,* or *D. multivorans*, we can assume that the even higher trace element concentrations in the water droplets did not limit the enzyme FDH *in situ* and were thus not responsible for the high formate concentrations in the water droplets. Interestingly, the selenium concentration in the pooled water droplets was so high that it could potentially inhibit sulfate reduction *in situ*.

Formate as inhibitor of sulfate reduction

To investigate a possible inhibition of sulfate reduction by formate and to get a better understanding of the physiological mechanisms behind formate inhibition, we performed formate inhibition experiments with three sulfate-reducing bacterial strains differing in their energy metabolism: *D. vulgaris*, *D. curvatus*, and *D. multivorans*.

Effect of formate on sulfate reduction by *D. vulgaris*

Sulfate reduction rates were calculated for *D. vulgaris* from the logarithmic growth phase, i.e. from 6.8 h until 23 h. Interestingly, we observed the highest sulfate reduction in the control lacking the reducing agent Na-thioglycolate and ascorbic acid (reduction of 5.74 mM sulfate; Fig. 2A). Nevertheless, ANOVA combined with a Tukey *post-hoc* test did not reveal significant effects of formate on

sulfate reduction rate, the total amount of sulfate reduced, or the onset of sulfate reduction by *D. vulgaris* (Figs. 2A and 5, Tables S1– S3, Supporting Information). *D. vulgaris* had a specific growth rate of 2.3 $\pm$ 0.1 day^{-1} in the positive control (0 mM formate). In contrast to sulfate reduction, there was a significant effect of 8 mM formate on growth, leading to a 1.6-fold reduction of the specific growth rate (1.4 $\pm$ 0.2 day $^{-1}$) compared to the treatment with 0 mM formate (ANOVA ($F(4,10)$ = 254.9, $P < 0.001$; Tukey's post-hoc test $P_{adj} < 0.001$). Addition of 0.08 mM molybdate as control completely inhibited growth (Figs. 2B and 5C). The 2.5 mM formate treatment caused no significant changes in growth or sulfate reduction. Chloroform treatment slowed down the growth but did not affect sulfate reduction rate or the onset of sulfate reduction or growth. Formate was not oxidized by *D. vulgaris* and remained stable at 2.6 $\pm$ 0.11 and 8.26 $\pm$ 0.25 mM (Table S1, Supporting Information; and supplementary raw data of *D. vulgaris*-formate). The pH-value of all cultures remained stable during the whole cultivation period of 28 h with a mean of 7.02 $\pm$ 0.35 ($n = 171$).

Effect of formate on sulfate reduction by *D. curvatus*

Sulfate reduction rates were calculated for *D. curvatus* from the logarithmic growth phase between day 3 and 6. One-way ANOVA with subsequent Tukey *post-hoc* tests revealed that 8 mM formate lead to a 2.4-fold decrease of the sulfate reduction rate to 0.987 $\pm$ 0.246 mM sulfate day^{-1}, compared to the treatment without formate with 1.934 $\pm$ 0.211 mM sulfate day^{-1} (ANOVA ($F(4,10)$ = 10.69, $P = 0.00124$, Tukey's $P_{adj} = 0.018$; Tables S1–S3, Supporting Information). Treatment with 0.08 mM molybdate slowed down sulfate reduction significantly (0.700 $\pm$ 0.214 mM day $^{-1}$, Tukey's $P_{adj} = 0.003$) but did not inhibit completely. The treatments with 2.5 mM formate and chloroform had no inhibitory effects (Tukey's $P_{adj} = 0.648$ and $P_{adj} = 0.993$). Effects on sulfate reduction induced by 8 mM formate and molybdate were similar. In the positive control (0 mM formate), *D. curvatus* had a specific growth rate of 1.9 $\pm$ 0.1 day^{-1}. The 2.5 and 8 mM formate treatments decreased the growth rates of *D. curvatus* significantly by a factor of 1.2 to 1.53 $\pm$ 0.06 day^{-1} (ANOVA ($F(4,10)$ = 74.64, $P < 0.001$; Tukey's P$_{adj} = 0.8 \times 10^{-2}$) and 1.8 to 1.03 $\pm$ 0.17 day^{-1} (Tukey's P$_{adj} < 0.001$) in contrast to the untreated positive control (1.88 $\pm$ 0.09 day^{-1}). The onset of growth was not affected by any treatment (Figs. 3B and 5D, Tables S1–S3, Supporting Information). Chloroform treatment had no effect on *D. curvatus*, while growth of the molybdate-treated cultures was slower (0.90 $\pm$ 0.02 day^{-1}, Tukey's P$_{adj} < 0.001$) compared to the positive control (Figs. 3B and 5C) and comparable to the growth rate of the 8 mM formate-treated cells. No growth was detected in the negative control (without electron-donor) or the sterile control. The pH-values remained stable during the whole experiment at 7.29 $\pm$ 0.26 ($n = 209$).

Effect of formate on sulfate reduction by *D. multivorans*

Sulfate reduction rates were calculated for *D. multivorans* from the linear sulfate reduction phase. The exact time frame had to be selected for each treatment separately because the onset of sulfate reduction, formate consumption, and growth of *D. multivorans* differed significantly between treatments (ANOVA ($F(5,9)$ = 8.08 $\times$ 10^{31}, $P < .001$; Figs. 4 and 5; and Tables S1–S3, Supporting Information). Treatments with 10 and 31 mM formate and molybdate were conducted in duplicates instead of triplicates. Addition of 10 and 31 mM formate lead to a 1.3-fold reduction in the sulfate
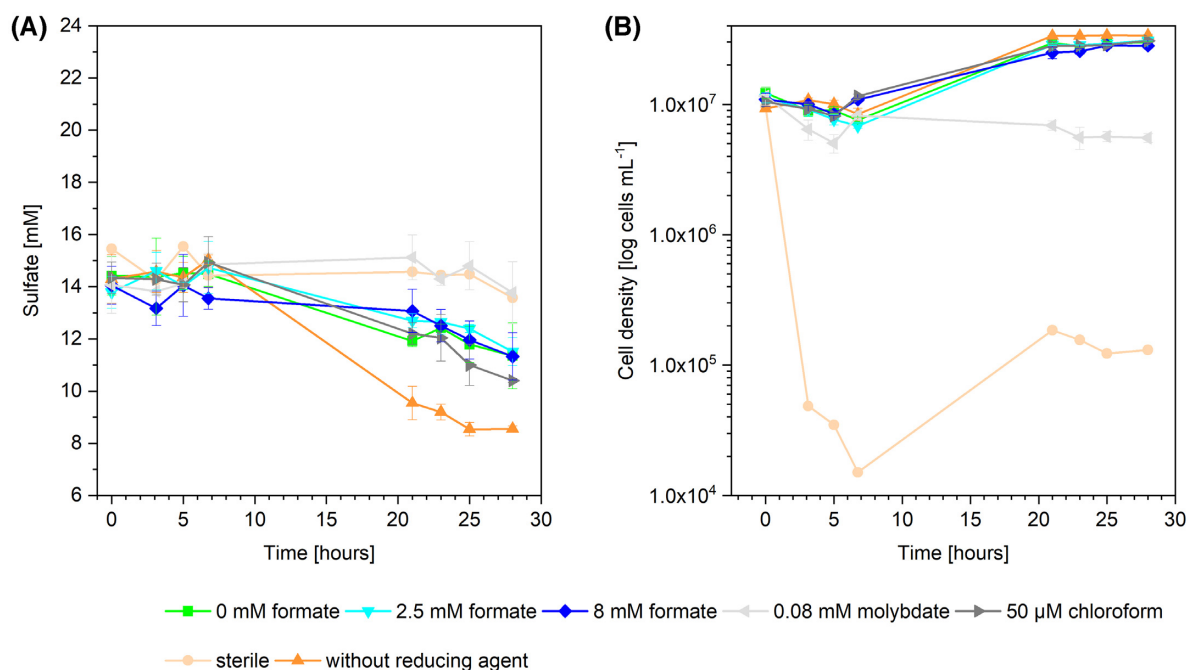
Figure 2. *Desulfovibrio vulgaris* cultivated with different concentrations of formate and with molybdate and chloroform. (**A**) Sulfate concentrations [mM], (**B**) cell densities [log cells mL^{-1}]. Symbols show means of triplicate incubations and vertical bars the standard deviation. Symbols for the sterile control represent single incubations.
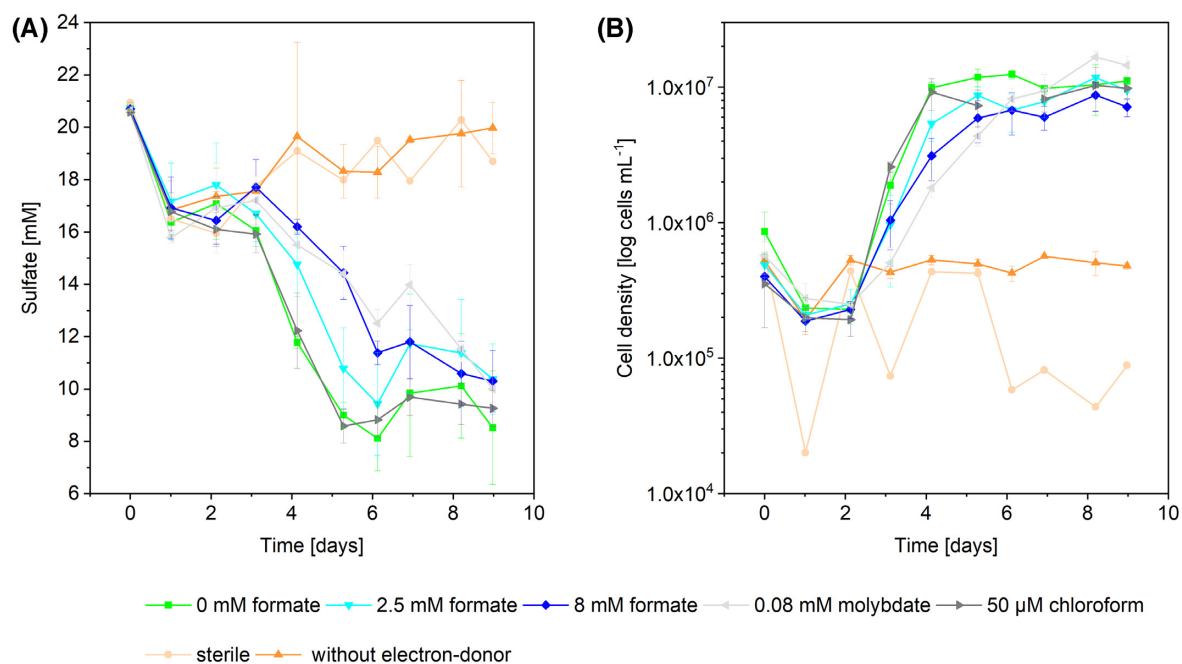

Figure 3. *Desulfobacter curvatus* cultivated with different concentrations of formate and with molybdate and chloroform. (**A**) Sulfate concentrations [mM], (**B**) cell densities [log cells mL^{-1}]. Symbols show means of triplicate incubations and vertical bars show the standard deviation. Symbols for the sterile control represent single measurements.

reduction rate compared to the untreated positive control with 0 mM formate, ANOVA ($F(5,9) = 86.21$, $P < 0.001$; Tukey *post-hoc* test: 10 mM formate: $P_{adj} = 0.03$; 31 mM formate: $P_{adj} = 0.004$). Sulfate reduction rate was not affected by 2.5 mM formate treatment (1.15 ± 0.1 mM day^{-1}, Tukey's $P_{adj} = 0.99$; Figs. 4A and 5A; and Tables S1–S3, Supporting Information). Increasing formate concentration lead to an increasingly strong delay in the onset

of sulfate reduction. Treatment with 10 and 31 mM formate delayed the sulfate reduction by 5 and 17 days (ANOVA ($F(5,9) = 243\,572$, $P < 0.001$, 10 mM formate Tukey's $P_{adj} = 0.03$, 31 mM formate Tukey's $P_{adj} < 0.001$), respectively, in contrast to the untreated control (0 mM formate). 2.5 mM formate did not affect the onset of sulfate reduction (Tukey's $P_{adj} = 1$; Fig. 5B and D; and Tables S1–S3, Supporting Information).
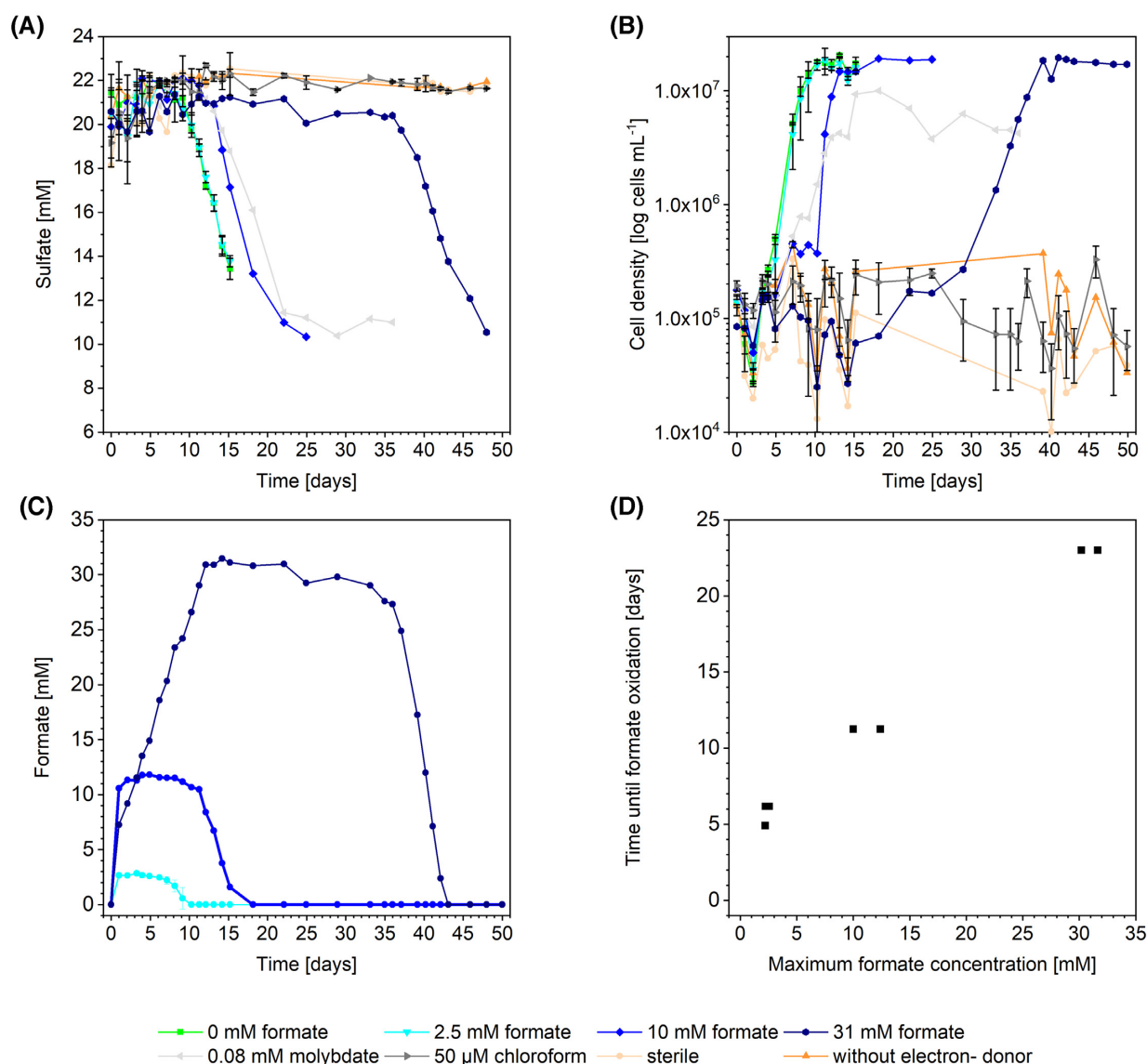
Figure 4. *Desulfococcus multivorans* cultivated with different concentrations of formate and with molybdate and chloroform. (**A**) Sulfate concentrations [mM], (**B**) cell density [log cells mL^{-1}], (**C**) formate concentrations [mM], (**D**) correlation between maximal formate concentration and the time point until formate oxidation started. Symbols for treatment with 0 and 2.5 mM formate and 50 μM chloroform show means of triplicate incubations, vertical bars show the standard deviation. Symbols for treatments with 10 and 31 mM formate and 0.08 mM molybdate show the mean of duplicate incubations. Symbols for the sterile control represent single measurements.

In the positive control, *D. multivorans* had a growth rate of 0.9 ± 0.008 h (Fig. 4B, Table S1, Supporting Information). The growth rate of *D. multivorans* was positively affected by increasing formate concentrations (up to 1.2 ± 0.3 day^{-1}, ANOVA ($F(5,9) = 48.72$, $P < 0.001$). However, this result is most likely caused by the significantly higher growth rate in the 10 mM formate treatment (Tukey's $P_{adj} = 0.03$), which might be a method artifact in cell counting between day 7 and 10 (see Fig. 4B, 10 mM formate graph). The growth rate was decreased by half in the 31 mM treatment (0.4 ± 0.04 day^{-1}, Tukey's $P_{adj} = 0.009$). Molybdate significantly lowered the growth rate and chloroform lead to a total inhibition of growth, as expected.

Interestingly, daily formate addition up to 31 mM formate completely inhibited sulfate reduction and growth of *D. multivorans*. However, after daily formate addition had ceased, growth took off approximately at day 22. Final cell densities were comparable to the positive control (0 mM formate), indicating no formate uti-

lization for active growth. Sulfate was not reduced in the sterile control or the negative control.

In contrast to *D. vulgaris* and *D. curvatus*, *D. multivorans* was able to oxidize formate *via* the WLP (Fig. 4C and D). Addition of 10 mM and 31 mM formate resulted into a three to two times higher formate oxidation rate (1.6 ± 0.4 and 3.7± 0.7 mM formate day^{-1}) compared to the formate oxidation rates in the 2.5 mM (0.5 ± 0.03 mM formate day^{-1}; ANOVA ($F(3,6) = 240.3$, $P < 0.001$; Tukey's *post-hoc* test 10 mM tested versus 2.5 mM $P_{adj} = 0.002$, Tukey's *post-hoc* test 31 mM tested versus 2.5 mM $P_{adj} < 0.001$) treated cultures (Tables S1–S3, Supporting Information). *D. multivorans* clearly started sulfate reduction only when the formate concentration was below 6.7 mM in the treatment with 10 mM formate (day 13) and when it was below 25 mM in the treatment with 31 mM (day 37). This suggests that sulfate reduction is not delayed by a specific formate threshold concentration. Instead, there seems to be a linear relationship between the maximal formate
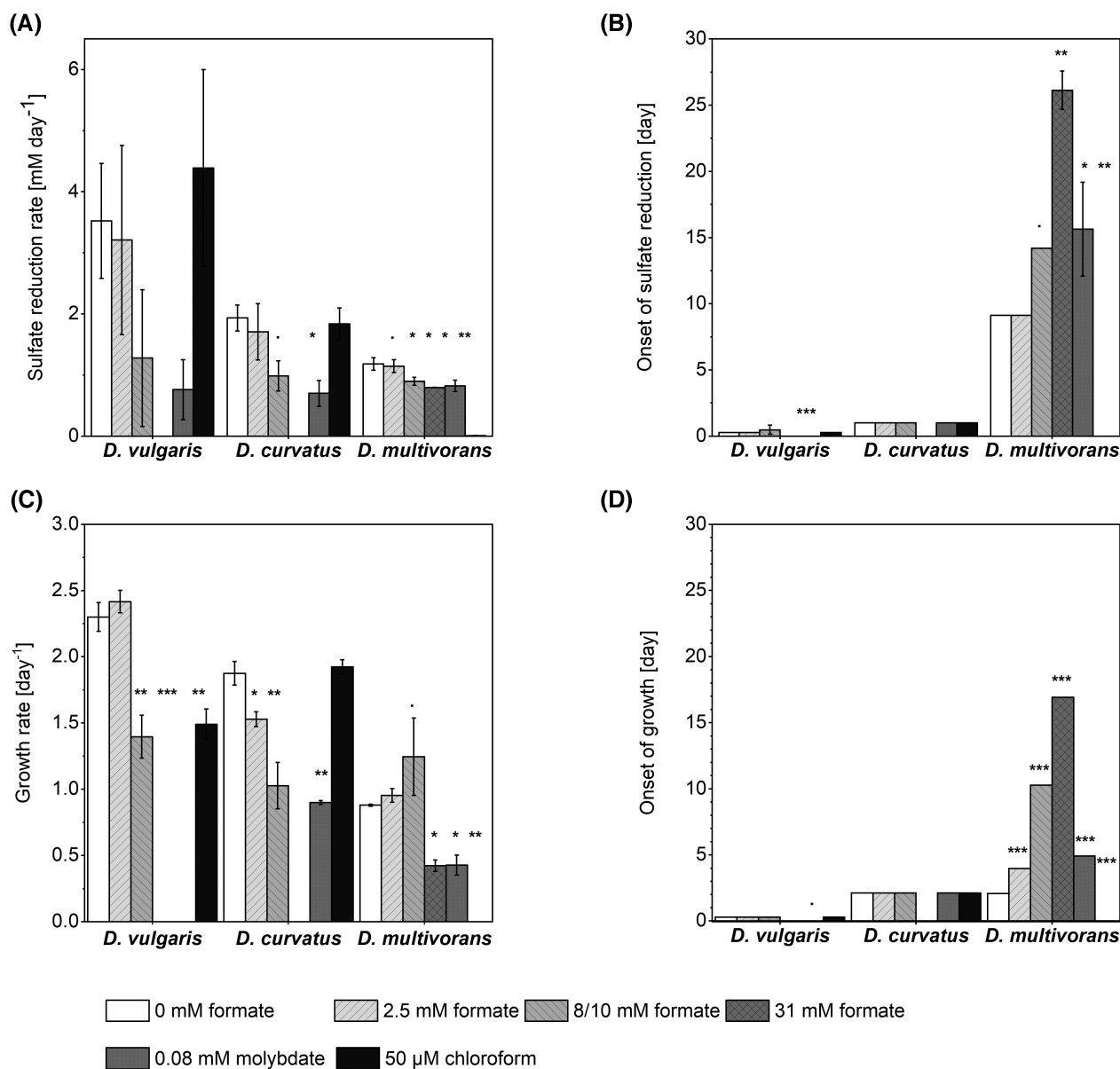
Figure 5. Effects of different formate concentrations, molybdate, and chloroform on *D. vulgaris*, *D. curvatus*, and *D. multivorans*. (**A**) Sulfate reduction rate, (**B**) onset of sulfate reduction, (**C**) growth rate, and (**D**) onset of growth. Stars above bars represent the significance of the treatment effects as tested by ANOVA with subsequent Tukey's *post-hoc* test (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; and ·$P \leq 0.1$).

concentration in the growth medium and the amount of time by which the onset of formate oxidation is delayed (Fig. 4D). The pH-value in all experiments remained stable at 7.4 ± 0.2 ($n = 466$) over the complete period.

Discussion

Methanogenesis is the major terminal electron-accepting process in produced oil reservoirs (Jones *et al.* 2008; Sherry *et al.* 2020). However, when sulfate is supplied by water injection to increase reservoir pressure, sulfate-reduction dominates because it is thermodynamically favorable over methanogenesis when sulfate concentrations exceed 50 μM (Jiménez *et al.* 2016). In this study, we studied the striking lack of sulfate-reducing microorganisms in the Pitch Lake water droplets despite high sulfate concentrations. To detect possible inhibitors of sulfate-reduction, we combined observations from the field with laboratory experiments on type strains of sulfate-reducing bacteria.

High experimental formate concentrations slow down and delay sulfate reduction of sulfate-reducing microorganisms with different energy metabolism

Depending on the type of central metabolism, formate treatment caused different effects on the sulfate reduction rate, growth rate, onset of sulfate reduction, and the beginning of cell growth. *D. multivorans* was most affected by formate treatment. The ability of formate utilization depends mostly on the enzyme FDH, which oxidizes formate to CO_2 (da Silva *et al.* 2013). Interestingly, in our study, *D. multivorans* was the only organism, which consumed formate despite the fact that the strain was described to be unable to utilize formate physiologically and genetically, due to missing genes encoding FDH (Dörries *et al.* 2016). We also observed that the onset of formate utilization and sulfate reduction by *D. multivorans* were delayed with higher initial formate concentration. Maybe, *D. multivorans* encodes other genes with related function

to FDH, or previous gene assembly or gene annotation may simply have missed out FDH (Warren *et al.* 2010).

D. multivorans did not use formate for growth, since the formate oxidation started mainly at the late logarithmic growth phase and almost simultaneously with the delayed sulfate reduction. Sulfate reduction seemed to be coupled to the oxidation of formate, as already known for some sulfate-reducing organisms (Ragsdale 2007). The cell density did not increase compared to the controls with benzoate only, suggesting no additional formation of biomass. This might indicate that formate was used for production of storage compounds such as polyhydroxyalkanoates (PHAs), which usually occurs in the late exponential to stationary phase. PHA-synthesis is known for *D. multivorans* (Hai *et al.* 2004, Ren *et al.* 2010), but we did not test PHA-production, here. Alternatively, formate may have been metabolized for detoxification rather than for growth, which would be consistent with the previously reported observation that the strain is not able to utilize formate as growth substrate (Dörries *et al.* 2016). Very interesting remains the delayed growth of *D. multivorans* upon formate exposure. It appears that the strain was somehow resilient to formate and was able to start growth after a certain time of formate exposure and oxidation. This resilience increased with every additional treatment, as the onset of formate oxidation was not fixed to a specific formate concentration but was more likely affected by a linear relationship between the maximal conducted formate concentration and the beginning of formate oxidation. This might reflect an adaptation mechanism such as the upregulation of gene products to metabolize the added formate.

D. vulgaris is described to encode three periplasmic but no cytoplasmic FDH's and should be able to use formate as electron-donor (Heidelberg *et al.* 2004, Zhang *et al.* 2006, da Silva *et al.* 2013). Thus, we did not expect an inhibitory effect of formate, nevertheless, *D. vulgaris* did not oxidize formate in our experiments. *D. vulgaris* preferred lactate as electron-donor instead, which might be due to some kind of catabolite repression.

D. curvatus did not oxidize formate, which agrees to a study, which did not detect FDH activity for *D. curvatus* (Ekstrom, Morel, Benoit 2003). Sulfate reduction and growth rate were significantly slowed down in the 8 mM formate treatment and resulted in comparable inhibition as with molybdate treatment.

Even though 0.08 mM molybdate should inhibit sulfate reduction (de Jesus *et al.* 2015), we could only achieve a full inhibition of sulfate reduction for *D. vulgaris* but not for *D. curvatus* or *D. multivorans*, for which the sulfate reduction rate was only slowed down. During the experiments, we observed a growth medium color change from transparent to gradually more yellow-brownish in all molybdate-treated cultures of *D. curvatus* and *D. multivorans*. Most likely, molybdenum–disulfide complexes were built, which resulted in the removal of the toxic molybdate (Biswas *et al.* 2009) prohibiting a complete inhibition of sulfate reduction.

Indications of formate inhibition of sulfate-reducing microorganisms in Pitch Lake water droplets

In the absence of sulfate, many sulfate-reducing microorganisms are capable of switching their metabolism to fermentation (Gieg, Fowler, Berdugo-Clavijo 2014), whereof formate, succinate, acetate, lactate, and ethanol are important products (Glombitza *et al.* 2015). Formate can also serve as electron donor for sulfate reducers or methanogens and many other microorganisms (da Silva *et al.* 2013). In most environmental settings, formate is consumed by methanogens leading to constant natural formate concentra-

tions below 10 µM (Sieber, McInerney, Gunsalus 2012, Glombitza *et al.* 2015). For example, constant environmental concentrations of formate (4–6 µM) have been observed throughout a sulfate reduction zone in sub-arctic sediments of Greenland (Glombitza *et al.* 2015). In view of these results, it is surprising that we found such high and constant concentrations of formate at surprisingly high concentrations of around 2.37 ± 0.05 mM in half of the investigated Pitch Lake water droplets. The special setting of the water-droplets embedded in oil might lead to these exceptional formate concentrations by so far unknown factors. Although the formate concentrations in our water droplets were 1000-fold higher, this indicates that our observation of formate in environmental samples is not exclusive to the Pitch Lake water droplets and that formate concentration in anoxic environments might happen more frequently and should be considered to be investigated in more detail.

The results of our experiments on the three type strains provide only partial support for our initial hypothesis, that the high *in situ* concentrations of 2.5 mM formate might have inhibited the sulfate-reducing bacteria in the Pitch Lake water droplets, because experimental inhibitory effects were observed only for higher formate concentrations of 8, 10, or 31 mM. Yet, in the natural environment of the Pitch Lake, cells are exposed to multiple stressors, and thus, 2.5 mM formate might already be sufficient to inhibit sulfate-reducing microorganisms. For instance, the saturated oil concentrations in the water phase may pose an additional challenge to the microbial activity or membrane integrity and enhance a decoupling effect of formate (Sikkema, de Bont, Poolman 1995, Li *et al.* 2019). Moreover, membrane integrity might be challenged *in situ* because temperatures in the Pitch Lake reach up to 58.3°C and are thus much higher compared to our laboratory experiments. Elevated temperatures result in higher membrane fluidity (Los, Murata 2004), which again might render the cells more susceptible to decoupling of the membrane potential by formate. This hypothesis is supported by a study on anaerobic sludge bed reactors with continuous supply of formate as main growth substrate at growth temperatures between 65 and 75°C. In this study, growth of methanogens was favored over growth of sulfate-reducing bacteria (Vallero *et al.* 2004). Although sulfate-reducing bacteria usually outcompete methanogens because they can reach lower residual substrate concentrations, the performance of the methanogens could be explained by sulfate reducers being inhibited by high formate concentrations. Yet, Vallero *et al.* (2004) explained their observation by the strong competition between sulfate-reducing bacteria and methane-producing archaea for formate or the H_2S toxic shock.

We observed that formate concentrations in the Pitch Lake water droplets seem to be under microbial control, i.e. kept at constant concentrations, and that formate had negative effects on the three type strains. This endorses the hypothesis that high *in situ* formate concentrations inhibit the sulfate-reducing microorganisms in the Pitch Lake water droplets and that methanogens might be favored over sulfate-reducing bacteria. At first glance, these results seem to contrast our previous study where sulfate-reducing organisms could be enriched in microcosms (Pannekens *et al.* 2021). However, the microbial communities in the microcosm experiments were cultivated in close to natural but still artificial medium where formate or selenium were not present at high concentrations. A potential inhibitor, which might have remained in the oil, was most likely diluted by the addition of the artificial medium.

In addition to formate, also the high *in situ* selenium concentrations that we found in the Pitch Lake water droplets might lead to

inhibition of sulfate reduction, since selenate (SeO$_4^{2-}$) and selenite (SeO$_3^{2-}$) are known structural analogues of sulfate and sulfite, and can function as specific inhibitors of sulfate reduction (Stoeva and Coates 2019). However, without analysing the speciation and bio-availability of selenium species in the Pitch Lake water we can only speculate on the presence of selenium. Hence, reliable conclusions about inhibition by selenate are impossible at this stage. In the water droplets, however, we could exclude molybdate as an inhibitor of sulfate-reduction since molybdenum concentrations in the Pitch Lake were comparable to the growth medium concentrations. Similarly, nickel concentrations in the water droplets were unlikely to limit the FDH although too high nickel concentrations might be toxic for microorganisms (Macomber and Hausinger 2011). Potential nickel toxicity or microbial resistance mechanisms were not further investigated, neither for the Pitch Lake water droplets, nor for the tested bacterial strains. It would be of great importance if scientists could spent more attention on the natural formate abundance in environmental samples in their future studies. This would reveal whether such high and constant concentrations as detected in this study are restricted to extreme environments such as oil, or whether they have so far simply been overseen.

Conclusion

The experimental results support our hypothesis that sulfate-reducing microorganisms in the Pitch Lake water droplets are likely inhibited by high and constant formate concentrations. This would explain the surprisingly high *in situ* sulfate concentrations observed in the water droplets and the low abundance of sulfate-reducers in the natural microbial communities. Thus, sulfate-reducing microorganisms might play a minor role in oil degradation in the Pitch Lake compared to methanogens. Our findings also indicate a potential application of formate in oil and gas production as environmentally friendly and natural inhibitor of sulfate-reduction. Adding formate in the gas or oil production process might decrease production of harmful sulfite, reduce corrosion, and minimize financial losses. However, the inhibition by formate depends on the metabolism and the capability of formate oxidation of the microorganism under the respective living conditions.

Supplementary data

Supplementary data are available at *FEMSEC* online.

Acknowledgments

Conflicts of interest. None declared.

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