Inhibition of sulfate-reducing bacteria with formate

L. Voskuhl, D. Brusilova, V. S. Brauer and R. U. Meckenstock*

Faculty of Chemistry, Environmental Microbiology and Biotechnology (EMB), Aquatic Microbiology, University of Duisburg-Essen, Universitätsstr. 5, 45141 Essen, Germany

*Corresponding author: Faculty of Chemistry, Environmental Microbiology and Biotechnology (EMB), Aquatic Microbiology, University of Duisburg-Essen, Universitätsstr. 5, 45141 Essen, Germany. Tel. +49 (0201) 183 6601, E-mail: rainer.meckenstock@uni-due.de

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.

Keywords: oil degradation, SRB, Pitch Lake, D. multivorans, D. vulgaris, D. curvatus

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 non-hazardous to human health-chemical to prevent corrosion 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 ± 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, Desulfuromonadaceae, Noridesulfospiraceae, 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 Oliveira 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 Sylvia 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 rela-
tive abundances of methanogenic archaea and marker genes as-
associated with methanogenesis (Schulze-Makuch et al. 2011, Meck-
enstock et al. 2014). Interestingly, however, sulfate-reducing mi-
croorganisms from the Pitch Lake were successfully enriched in
an experiment where Pitch Lake oil containing the indigenous mi-
crobiota 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 inhib-
ited by oil reservoir chemicals or microbial metabolites in the wa-
ter droplets.

While analysing ion compositions to characterize the geochem-
ical conditions of individual water droplets (Voskuhl et al. 2021),
we noticed exceptionally high formate concentrations never re-
ported 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 ac-
cumulated in *Escherichia coli* cultures during mixed acid fer-
tmentation and that concentrations of 20 mM formate delayed the
growth of *E. coli* by acidic stress, causing decoupling of the mem-
brane potential (Kirkpatrick et al. 2001). Formate is a common fer-
tmentation product (Knapp and Sawers 1990, Crable et al. 2011)
and may accumulate in the surroundings of cells, provoking inhib-
itory effects at high concentrations. However, formate may not
only have negative effects, but it may also serve as a growth sub-
strate that can be oxidized to CO₂ by the periplasmic enzyme for-
mate dehydrogenase (FDH). The gained electron equivalents can
be used for respiration. Formate can also be used for carbon fix-
ation via, e.g. the Wood–Ljungdahl pathway (WLP; Crable et al.
2011).

In this study, we investigated whether formate may inhibit sul-
fate reduction. In a first step, we measured formate concentra-
tions in 43 water droplets from the Pitch Lake. We also deter-
mined the in situ availability of molybdenum, nickel, and sele-
nium, 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 in-
hibitors of sulfate reduction (Stoeva and Coates 2019). In a sec-
ond step, generic inhibition by formate was studied by treating three
sulfate-reducing bacterial strains with formate concentra-
tions 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 lac-
tate oxidation (Heidelberg et al. 2004, Pereira et al. 2008), *Desulfo-
bacter curvatus*, performing acetate degradation via the citric acid cy-
cle (TCA; Ekstrom, Morel and Benoit 2003), and *Desulfooccus multi-
vorans*, 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 mi-
crobial 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 ~ 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 stable isotopes have
proven that the water droplets originate from the deep oil reser-
voir, implying that the microbial communities therein originate
from the deep reservoir as well (Meckenstock et al. 2014). In or-
der to isolate the water droplets from the oil, we first sampled oil
from the Pitch Lake surface, transferred it into sterile, glass 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 Desulfovolutibactera (formerly Deltaproteobacteria; Waite et al.
2020), namely *Desulfovibrio vulgaris* (DSM 644), *Desulfofer curva-
tus* (DSM 3379), and *Desulfooccus multiivorans* (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. multi-
vorans). 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 *Desul-
oflactibacteraceae*, which includes the strains D. curvatus and D. multi-
vorans, 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. cur-
vatus with medium DSM 193 at 30°C. D. multivorans and D. curva-
tus were incubated on a shaker at 150 rounds per minute (rpm)
to avoid flocculation of the cells. Cultures were incubated un-
der 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% N₂ and
20% CO₂ (BIOGON® C20, Linde, Germany), while cultures of D. vul-
garis were flushed with pure nitrogen. The medium for D. curvatus
and D. multivorans contained 20 mM sulfate and 15 mM for D. vul-
garis. D. vulgaris was grown with 18 mM Na-DL-lactate, D. curva-
tus 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 sam-
ples in the stationary phase and inoculated 1:10 into freshly pre-
spared medium DSM 63, DSM 193, and DSM 195, respectively. Ini-
tial cell numbers were about 1 x 10⁹ mL⁻¹ for D. curvatus and D. multi-
vorans and about 1 x 10⁷ 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 follow-
ing treatment conditions: (I) 0 mM formate, positive control; (II)
2.5 mM formate; (III) 8 mM (D. vulgaris and D. curvatus), D. multi-
vorans was unintentionally cultivated with 10 mM formate; (IV)
0.08 mM molybdate as an 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 ± 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 MΩ cm, 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 C18 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 NaHCO3 and 4.5 mM Na2CO3 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 SRS, Thermo Scientific) was applied for peak detection. 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 10000 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% HNO3, suprapure). Before analyses, samples were diluted 1:2 (DSM193) and 1:200 (Pitch Lake water droplet pool) with 1% HNO3 (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 (50 H Elmsionic, 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 analyzed 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
droplets, formate concentrations were above the detection limit and showed a mean of 2.37 mM formate ± 0.05 mM (Fig. 1). To investigate if a potential lack of molybdenum, nickel, or selenium may accumulate the concentration 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 ± 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 ± 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 ± 0.11 and 8.26 ± 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 ± 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 ± 0.246 mM sulfate day⁻¹, compared to the treatment without formate with 1.934 ± 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 ± 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 ± 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 ± 0.06 day⁻¹ (ANOVA (F(4,10) = 74.64, P < 0.001; Tukey’s P adj = 0.8 × 10⁻²) and 1.8 to 1.03 ± 0.17 day⁻¹ (Tukey’s P adj < 0.001) in contrast to the untreated positive control (1.88 ± 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 ± 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 ± 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 × 10⁻¹³, 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
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: Padj = 0.03; 31 mM formate: Padj = 0.004). Sulfate reduction rate was not affected by 2.5 mM formate treatment (1.15 ± 0.1 mM day⁻¹, Tukey’s Padj = 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 Padj = 0.03, 31 mM formate Tukey’s Padj < 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 Padj = 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⁻¹ (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 = 0.099). 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 utilization 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
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.

[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 ≤ 0.001; *P ≤ 0.01; P ≤ 0.05; and P ≤ 0.1).]

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 CO₂ (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...
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 (Dorries 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 concentrations below 10 μM (Sieber, McNerney, 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 the 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 of 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 H2S 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 (Fannekens 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 (SeO₄²⁻) and selenite (SeO₃²⁻) are known structural analogues of sulfate and sulfite, and can function as specific inhibitors of sulfate reduction (Stoeya 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 Hauinger 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 spend 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

This work was funded by the German Research Foundation (DFG; grant number BR 5493/1–1) and the European Research Council (ERC; grant number 666952–EcOILogy). We would like to thank Astrid Dannehil, Isabelle Heker, and Meike Arnold for help with anaerobic cultivation and Mark Pannekens for discussions on the sulfate-reduction potential in the Pitch Lake.

Conflicts of interest. None declared.

REFERENCES

Attwooll, AW, Broome, DC. Trinidad Lake Asphalt. London: The Baynard Press, 1954.
Biswas, KC, Woodards, NA, Xu, H et al. Reduction of molybdate by sulfate-reducing bacteria. Biometals 2009;22:131–9.
Bungert, D, Maikranz, S, Sundermann, R et al. The evolution and application of formate brines in high-temperature/high-pressure operations. In: Proceedings of the IADC/SPE Drilling Conference, New Orleans, LA: Society of Petroleum Engineers, 2000, 11. DOI: 10.2118/59191-MS.
Chen, Z, Yang, G, Hao, X et al. Recent advances in microbial capture of hydrogen sulfide from sour gas via sulfur-oxidizing bacteria. Eng Life Sci 2021;21:1–16.
Chilingarian, GV, Yen, TF. Bitumens, Asphalts and Tar Sands (Developments in Petroleum Science). Amsterdam; New York: Elsevier Science Ltd, 1979.
Crable, BR, Plugge, CM, McInerney, MJ et al. Formate formation and formate conversion in biological fuels production. Enzyme Res 2011;2011:532536.
da Silva, ML, Soares, HM, Furigo, A et al. Effects of nitrate injection on microbial enhanced oil recovery and oilfield reservoir souring. Appl Biochem Biotechnol 2014;174:1810–21.
da Silva, SM, Voordouw, J, Leițăo, C et al. Function of formate dehydrogenases in Desulfovibrio vulgaris hildenborough energy metabolism. Microbiology 2013;159:1760–9.
de Jesus, EB, de Andrade Lima, LRP, Bernardes, LA et al. Inhibition of microbial sulfate reduction by molybdate. Brazil J Petrol Gas 2015;9:95–106.
Di Martino, P. Ways to improve biocides for metalworking fluid. AIMS Microbiol 2021;7:13–17.
Dörries, M, Wöhlbrand, L, Kube, M et al. Genome and catabolic sub-proteomes of the marine, nutritionally versatile, sulfate-reducing bacterium Desulfococcus multivorans DSM 2059. BMC Genomics 2016;17:918.
Downs, JD. Formate brines: novel drilling and completion fluids for demanding environments. In: Proceedings of the SPE International Symposium on Oilfield Chemistry, New Orleans, LA: Society of Petroleum Engineers, 1993, 13. DOI: 10.2118/25177-MS.
Ekstrom, EB, Morel, FM, Benoit, JM. Mercury methylation independent of the acetyl-coenzyme a pathway in sulfate-reducing bacteria. Appl Environ Microbiol 2003;69:5414–22.
Gieg, LM, Duncan, KE, Sulfita, JM. Bioenergy production via microbial conversion of residual oil to natural gas. Appl Environ Microbiol 2008;74:3022–9.
Gieg, LM, Fowler, SJ, Berdugo-Clavijo, C. Syntrophic biodegradation of hydrocarbon contaminants. Curr Opin Biotechnol 2014;27:21–9.
Glombitza, C, Jaussi, M, Roy, H et al. Formate, acetate, and propionate as substrates for sulfate reduction in sub-Arctic sediments of southwest Greenland. Front Microbiol 2015;6:846.
Hai, T, Lange, D, Rabus, R et al. Polyhydroxyalkanoate (PHA) accumulation in sulfate-reducing bacteria and identification of a class III PHA synthase (PhaEC) in Desulfococcus multivorans. Appl Environ Microbiol 2004;70:4440–8.
Halic, A, Guhner, R, Watkin, E. Preliminary study on nitrate injection to control souring problem in oil reservoir: benefits and side effects on steel material. In: Proceedings of CORROSION 2011 Conference. Houston, Texas, 2011.
Heidelberg, JF, Seshadri, R, Haverman, SA et al. The genome sequence of the anaerobic, sulfate-reducing bacterium Desulfovibrio vulgaris hildenborough. Nat Biotechnol 2004;22:554–9.
Jiménez, N, Richnow, HH, Vogt, C et al. Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *J Mol Microbiol Biotechnol* 2016;26:227–42.

Jones, DM, Head, IM, Gray, ND et al. Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* 2008;451:176–80.

Kirkpatrick, C, Maurer, LM, Oyelakin, NE et al. Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. *J Bacteriol* 2001;183:6466–77.

Knappe, J, Sawers, G. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate-formate-lyase system of *Escherichia coli*. *FEMS Microbiol Rev* 1990;6:383–98.

Li, X, Qu, C, Bian, Y et al. New insights into the responses of soil microorganisms to poly cyclic aromatic hydrocarbon stress by combining enzyme activity and sequencing analysis with metabolomics. *Environ Pollut* 2019;255:113312.

Liu, Z-h, Yin, H, Lin, Z et al. Sulfate-reducing bacteria in anaerobic bio-processes: basic properties of pure isolates, molecular quantification, and controlling strategies. *Environ Technol Rev* 2018;7:46–72.

Los, DA, Murata, N. Membrane fluidity and its roles in the perception of environmental signals. *Biochim Biophys Acta Biomembranes* 2004;1666:142–57.

Macomber, L, Hausinger, RP. Mechanisms of nickel toxicity in microorganisms. *Metallomics* 2011;3:1153–62.

Martínez Arbizu, P. pairwiseAdonis: pairwise multilevel comparison using adonis. R package version 0.4. 2020. https://github.com/pmartinezarbizu/pairwiseAdonis.

McDowall, JS, Murphy, BJ, Haumann, M et al. Bacterial formate hydrogenlyase complex. *Proc Natl Acad Sci* 2014;111:E3948.

Meckenstock, RU, von Netzer, F, Stumpf, C et al. Water droplets in oil are microhabitats for microbial life. *Science* 2014;345:673–6.

Muyzer, G, Stams, AJ. The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* 2008;6:441–54.

Oksanen, J, Blanchet, FG, Friendly, M et al. vegan: community ecology package. R package version 25-7. 2020. https://CRAN.R-project.org/package=vegan.

Ostapkowicz, J, Brock, F, Wiedenhoef, AC et al. Black pitch, carved histories: radiocarbon dating, wood species identification and strontium isotope analysis of prehistoric wood carvings from Trinidad’s Pitch Lake. *J Archaeol Sci Rep* 2017;16:341–58.

Pannekens, M, Kroll, L, Müller, H et al. Oil reservoirs, an exceptional habitat for microorganisms. *New Biotechnol* 2019;49:1–9.

Pannekens, M, Voskuhl, L, Meier, A et al. Densely populated water droplets in heavy-oil seeps. *Appl Environ Microbiol* 2020;86:e00164–20.

Pannekens, M, Voskuhl, L, Mohammadian, S et al. Microbial degradation rates of natural bitumen. *Environ Sci Technol* 2021;55:8700–8.

Pereira, PM, He, Q, Valente, FM et al. Energy metabolism in *Desulfovibrio vulgaris hildenborough*: insights from transcriptome analysis. *Antonie Van Leeuwenhoek* 2008;93:347–62.

Popoola, LT, Grema, AS, Latinwo, GK et al. Corrosion problems during oil and gas production and its mitigation. *Int J Ind Chem* 2013;4:35.

Ragsdale, SW. Nickel and the carbon cycle. *J Inorg Biochem* 2007;101:1657–66.

Ren, Q, de Roo, G, Witholt, B et al. Influence of growth stage on activities of polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida*. *U. B. Microbiol Microbiol* 2010;10:254.

Rossmore, HW, Sondossi, M. Applications and mode of action of formaldehyde condensate biocides. *Vol. 33*. In: Al Laskin (ed.). *Advances in Applied Microbiology*. Cambridge, MA: Academic Press, 1988, 223–77.

Schulze-Makuch, D, Haque, S, Antonio, MRD et al. Microbial life in a liquid asphalt desert. *Astrobiology* 2011;11:241–58.

Sherry, A, Grant, RJ, Aitken, CM et al. Methanogenic crude oil-degrading microbial consortia are not universally abundant in anoxic environments. *Int Biodeter Biodegrad* 2020;155:105085.

Sieber, JR, McInerney, MJ, Gunsalus, RP. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Annu Rev Microbiol* 2012;66:429–52.

Sierra-Garcia, NI, de Oliveira, VM. Microbial hydrogen degradation: efforts to understand biodegradation in petroleum reservoirs. In: *Biodegradation - Engineering and Technology*. IntechOpen. 2013. DOI: 10.5772/55920.

Sikkema, J, de Bont, JA, Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* 1995;59:201–22.

Simone, K, Gwinn, J, Neubauer, P. High cell density media for *Escherichia coli* are generally designed for aerobic cultivations – consequences for large-scale bioprocesses and shake flask cultures. *Microb Cell Fact* 2008;7:26.

Sondossi, M, Rossmore, HW, Williams, R. Relative formaldehyde resistance among bacterial survivors of biocide-treated metalworking fluid. *Int Biodeter Biodegrad* 2001;48:286–300.

Stoeva, MK, Coates, JD. Specific inhibitors of respiratory sulfate reduction: towards a mechanistic understanding. *Microbiology* 2019;165:254–69.

Valero, MV, Camarero, E, Lettinga, G et al. Thermophilic (55-65 degrees C) and extreme thermophilic (70-80 degrees C) sulfate-reducing bacteria. *Nat Rev Microbiol* 2008;6:441–54.

Voskuhl, L, Akhari, A, Müller, H et al. Indigenous microbial communities in heavy oil show a threshold response to salinity. *FEMS Microbiol Ecol* 2021;97:34864985. DOI: 10.1093/femsec/fiab157.

Waite, DW, Chuvchchina, M, Pelikan, C et al. Proposal to reclassify the proteobacterial classes Deltaproteobacteria and *Oligoflexia* lexia, and the phylum thermodesulfovibrioacteria into four phyla reflecting major functional capabilities. *Int J Syst Evol Microbiol* 2020;70:5972–6016.

Warren, AS, Archuleta, J, Feng, W-c et al. Missing genes in the annotation of prokaryotic genomes. *BMC Bioinf* 2010;11:131.

Xue, Y, Voordouw, G. Control of microbial sulfide production with biocides and nitrate in oil reservoir simulating bioreactors. *Biotechnol Progr* 2004;20:1382–92.

Yin, H, Lin, Z et al. Methane production and its mitigation: evidence from field and laboratory studies. *J Mol Microbiol Biotechnol* 2016;26:227–42.

Zhang, W, Culley, DE, Scholten, JCM et al. Global transcriptomic analysis of *desulfovibrio vulgaris* on different electron donors. *Antonie Van Leeuwenhoek* 2006;89:221–37.

Zhang, Z, Ni, M, He, M et al. Competition and cooperation of sulfate reducing bacteria and five other bacteria during oil production. *J Pet Sci Eng* 2021;203:108688.