

RESEARCH ARTICLE

Indigenous microbial communities in heavy oil show a threshold response to salinity

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One sentence summary: Microbial communities from Pitch Lake oil show a sudden threshold-regulated response to salinity, leading to less diverse and uneven communities

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ABSTRACT

Microbial degradation influences the quality of oil resources. The environmental factors that shape the composition of oil microbial communities are largely unknown because most samples from oil fields are impacted by anthropogenic oil production, perturbing the native ecosystem with exogenous fluids and microorganisms. We investigated the relationship between formation water geochemistry and microbial community composition in undisturbed oil samples. We isolated 43 microliter-sized water droplets naturally enclosed in the heavy oil of the Pitch Lake, Trinidad and Tobago. The water chemistry and microbial community composition within the same water droplet were determined by ion chromatography and 16S rRNA gene amplicon sequencing, respectively. The results revealed a high variability in ion concentrations and community composition between water droplets. Microbial community composition was mostly affected by the chloride concentration, which ranged from freshwater to brackish-sea water. Remarkably, microbial communities did not respond gradually to increasing chloride concentration but showed a sudden change to less diverse and uneven communities when exceeding a chloride concentration of 57.3 mM. The results reveal a threshold-regulated response of microbial communities to salinity, offering new insights into the microbial ecology of oil reservoirs.

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INTRODUCTION

Oil reservoirs are extreme habitats for microorganisms with extremely variable salinity ranges, high temperatures (50–131°C), high toxicity and a low water activity of the oil (Röling, Head and Larter 2003; Pannekens *et al.* 2019). Nevertheless, adapted microorganisms are able to thrive not only at the oil-water transition zone below the reservoir but also dispersed in microliter-sized water droplets within the oil leg (Röling, Head and Larter 2003; Kim and Crowley 2007; Meckenstock *et al.* 2014; Pannekens *et al.* 2020). The activity of microorganisms regulates the natural biodegradation of oil in reservoirs and reservoir souring during oil production, and therefore, has generated interest in understanding the environmental factors that determine the assembly of microbial communities in oil (Youssef *et al.* 2009; Arora *et al.* 2016).

How environmental conditions shape autochthonous microbial communities in oil reservoirs is largely unknown (Magot 2005; Michas *et al.* 2017; Vigneron *et al.* 2017). Firstly, it is often challenging to unravel the real effects of specific variables such as salinity from other environmental variables due to environmental complexity. Secondly, the understanding of microbial community assembly in oil is hampered by the expensive and logistically complex sampling through drilling. Hence, most samples stem from oil wells and are almost exclusively available from oil production (Kim and Crowley 2007; Gao *et al.* 2016; Vigneron *et al.* 2017; Shlimon *et al.* 2020). Drilling and water injections, though, perturb the native ecosystem with electron acceptors such as oxygen and sulfate, nutrients and exogenous microbes (Head, Jones and Larter 2003; Aitken, Jones and Larter 2004). Only a few studies have investigated the impact of environmental factors in undisturbed oil samples. Kim and Crowley (2007) characterized samples from two asphalt soil mixes of the Rancho La Brea tar pits in California and found different geochemical conditions and different microbial communities in the samples. The small sample size prevented the statistical examination of a likely relationship between water chemistry and community composition. Also, Gao *et al.* (2016) reported geochemical conditions and microbial community compositions in two non-water flooded oil reservoirs, which were compared to several water-flooded reservoirs across China. Yet, anthropogenic impacts by oil production likely affected the non-flooded reservoirs, and the small number of potentially undisturbed reservoirs did not allow to draw general conclusion on the effects of geochemical parameters on community composition either. More detailed insight on anthropogenic and environmental impacts on oil microbiomes was provided by Vigneron *et al.* (2017), who analysed microbial succession from two sites of the Halfdan oil field in the North Sea during the first 15 years of oil field development. The initial, presumably native microbial community was influenced by the geochemistry of the formation water, in particular salinity. Therefore, previous studies are either limited in sampling size or by operationally disturbed samples. An extensive examination of truly undisturbed oil environment is yet to be explored.

To obtain a better understanding on the influence of environmental conditions on truly indigenous microbial communities, we here investigated microbial communities that can be found in μL -sized water droplets enclosed in the heavy oil matrix of the Pitch Lake, the worlds' largest natural asphalt seep located in Trinidad and Tobago (Fig. 1). Previous studies

have shown with the help of stable isotope analysis that the water droplets originate from the reservoir deep below the Pitch Lake, and that therefore, they represent undisturbed samples of native microbial communities consisting of anaerobic, metabolically active microorganisms (Meckenstock *et al.* 2014; Pannekens *et al.* 2020, 2021). To investigate the relationship between environmental conditions and microbial community composition we analysed 43 water droplets that we extracted from oil that we sampled at six different sites on the Pitch Lake. We characterized the environmental conditions in each water droplet with respect to the geochemistry of the water by measuring the concentrations of 13 inorganic ions, and determined the microbial community composition based on 16S rRNA gene amplicon sequences.

MATERIALS AND METHODS

Sampling and sample preparation

The Pitch Lake in La Brea, Trinidad and Tobago consists of natural asphalt and has an almost solid bitumen surface where one can walk on. Water can be found in puddles on the lake, but water is covering only small parts of the lake surface. Fresh, liquid oil is continuously rising from the subsurface to the lake surface at different locations (Fig. 1A–C). In the subsurface reservoir, there is the water leg beneath the oil leg and the boundary between those two legs is known as the oil-water-transition-zone (Bennett *et al.* 2013). This is the zone where the microbial degradation of hydrocarbon mostly occurs. The microbial communities that we investigated in this study live in tiny water droplets, which we extracted from oil that we sampled at six different sites on the surface of the Pitch Lake (Fig 1; Figure S1, Supporting Information). The tiny water droplets are most likely formed within the subsurface reservoir or during the ascent of the oil to the surface (Fig. 1A; Meckenstock *et al.* 2014). We thus assume that the microbial communities have a common origin in the oil reservoir. For this study, oil samples were taken on two consecutive days in March 2018. The different sampling sites in our study represent different oil seepages on the Pitch Lake surface (Fig. 1C and Figure S1, Supporting Information). Because the Pitch Lake is dynamic, oil seepages are temporary phenomena that open and close at different locations on the Pitch Lake (Brock *et al.* 2017; Ostapkowicz *et al.* 2017). This leads to a permanently changing lake surface and shoreline, prohibiting repeated sampling of the same sampling site on the long term. Sampling locations in this study were tracked via GPS signal and distances between sampling sites were calculated based on the GPS data (Figure S1, Supporting Information). Distances between sampling sites varied between 12 (sites 1 and 2) and 326 m (sites 4 and 6). The distance between individual water droplets within a sampling jar ranged from several millimeters to a few centimeters. Ambient air temperature ranged from 31.2 to 37.7°C, and temperature of the surface oil ranged from 37.1 to 58.3°C. As air and oil temperatures were strongly affected by incident solar irradiation (i.e. by daytime and cloudiness), we decided that these parameters were not useful for further interpretation of the data.

The oil was sampled with sterile, decapitated 50 mL syringes. The syringe was placed directly above the fresh, upcoming oil and dipped up to 15 cm into the liquid oil. The liquid but highly viscous oil was slowly soaked into the syringe and transferred to sterile glass jars (Fig. 1C). Glass jars were flushed with nitrogen



Figure 1. The Pitch Lake in Trinidad and Tobago and its water droplets. (A) Hypothetical scheme of the Pitch Lake. The magnification demonstrates how the water pockets may serve as habitats for the microbes within the porous oil leg. When the oil gets pressed up, water droplets are assumed to separate from the water pockets and rise, dispersed in the oil, to the surface of the Pitch Lake. During upwards flux, water droplets are expected to remain physically isolated and to be exposed to rather similar environmental conditions. (B) Aerial photograph of the Pitch Lake, covering approximately 47 hectares next to the Gulf of Paria. (C) Sampling of fresh, liquid oil at two oil seeps. Oil is gathered with decapitated syringes and transferred into sterile glass jars. (D) Extraction of a water droplet (indicated by the red arrow) from the glass jar containing oil by using a 10 μL pipette with corresponding tip size.

to establish an anoxic atmosphere. Shipment of anoxic samples needed 14 days, after which they were stored at 4°C, leading to solidification of the oil. For water droplet extraction, the glass jars containing the oil were heated to 45°C for 30 min to liquefy the oil. Due to their lower density, water droplets rose to the surface of the oil, where they were sampled with a 10 μL pipette (Fig. 1D). We extracted a total of 43 water droplets with a median volume of 3 μL and stored them at -70°C until further analyses. An overview over sampling day, sampling site and glass jar number corresponding to each water droplet is provided in Fig. 2 and Table S1 (Supporting Information).

Determination of microbial community compositions

A total of 1 μL of each water droplet was used for DNA-extraction, the remaining sample volume was preserved for ion chromatography. DNA extraction from 1 μL of sample was carried out according to a 2-step-lysis protocol (Pannekens et al.

2020). In short, DNA was treated 1 h at 37°C with 1 μL of a freshly prepared enzyme-mix, containing 2.5 U/ μL lysozyme, 0.048 U/ μL lysostaphin and 0.6 U/ μL mutanolysin in order to lyse a broad range of Gram-positive cells. This step was followed by an alkaline lysis of Gram-negative cells containing 0.4 M KOH and 0.1 M dithiothreitol. The alkaline lysis was stopped after 5 min reaction time by the addition of Tris-HCl (pH 4) (Pannekens et al. 2020). In order to rule out contamination of the samples by foreign DNA, negative controls were introduced at different steps during DNA extraction and 16S rRNA gene library preparation. Subsequent 16S rRNA gene amplicon sequencing of the V3–V4 region with forward primer Pro341F and reverse primer Pro805R (Takahashi et al. 2014) was performed in two technical replicates per droplet. Library preparation was accomplished according to the Illumina 16S metagenomic sequencing library preparation guide (Part # 15044223 Rev. B) with small modifications as previously described (Pannekens et al. 2020).

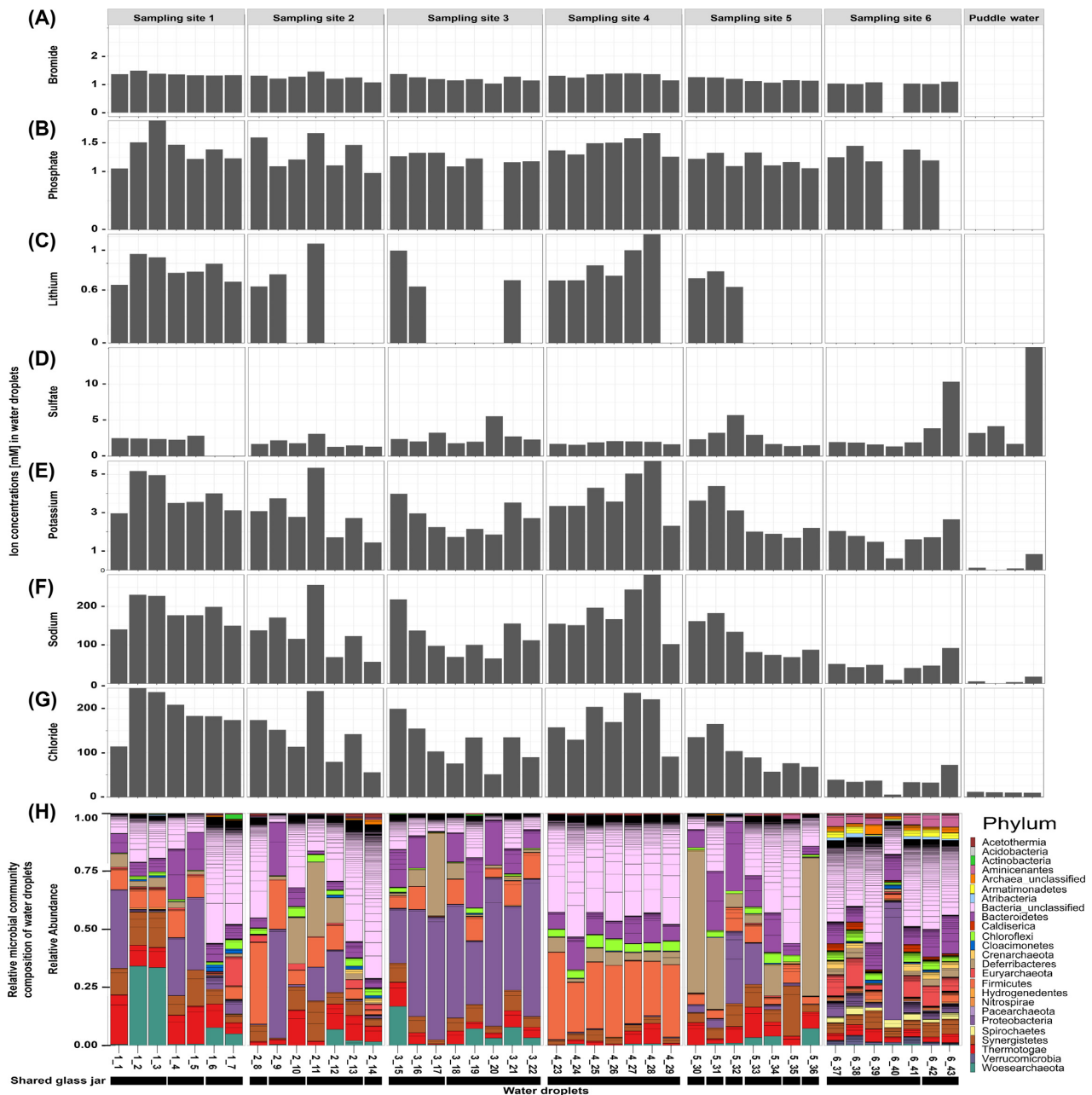


Figure 2. Ion concentrations and microbial community composition of 43 water droplets from six different sampling sites of the Pitch Lake. Ion concentrations of surface water puddles are shown for comparison in the right panel. Each bar represents the ion concentration or microbial community composition of a single water droplet, calculated as the mean of two technical replicates. (A)–(G) Concentrations of sulfate, phosphate, lithium, potassium, sodium, bromide and chloride in mM. Note different y-scales. (H) Relative abundance of OTUs clustered at a 97% sequence similarity level, with different colors representing different phyla, each casket represents a single OTU. Black bars at the bottom of the figure indicate the glass jar to which a water droplet belongs. Note that rare OTUs with very low abundances are not resolvable as single caskets with the bars and appear black. See Figure S3 (Supporting Information) for relative abundances of the Top 25 OTUs.

DNA sequence analysis was performed using mothur version 1.40.5 (Schloss et al. 2009). Sequences were clustered into operational taxonomic units (OTUs) with a 97% identity threshold. Quality filtering and read processing of the sequences of the 86 duplicated samples resulted in 2.6 million reads, with a mean read number of $67\,294 \pm 28\,183$. All samples were rarefied to the minimum of 15 728 reads. OTUs detected in only one water droplet or OTUs with a read number below 10 were rated as rare and removed from the dataset. Technical duplicates were merged by calculating the mean read number for each OTU.

Subsequent analyses of the microbial composition of the water droplet communities were performed with the R package phyloseq version 1.24.2 (McMurdie and Holmes 2013). Reclassification with BLAST NCBI (NCBI Resource Coordinators 2016) of 286 previously unclassified OTUs assigned most OTUs to *Dehalococcoidia* and the candidate phyla Atribacteria and Parcubacteria. A total of 581 OTUs was obtained, whereof 535 OTUs were assigned to 76 different genera from 19 bacterial phyla and 46 OTUs to four archaeal phyla (Fig 2H; Table S4, Supporting Information). Raw sequences are available on the NCBI database in Biopro-

ject PRJNA642708. The sequence data from sampling site 3 have already been published in Pannekens et al. (2020).

Quantitative PCR

In order to determine absolute cell numbers, we counted absolute copy numbers of the 16S rRNA gene by quantitative PCR (qPCR) following the protocol of Takai and Horikoshi (2000) with minor modifications. We amplified the V3–V4 region of the 16S rRNA gene of both bacteria and archaea using forward primer Uni340F (CCT ACG GGR BGC ASC AG), reverse primer Uni806R (GGA CTA CNN GGG TAT CTA AT) and the FAM-TAMRA-labelled Probe Uni516F (YCA GCM GCC GCG GTA AHA CVN RS). qPCR runs were performed in reaction volumes of 20 μL with 1 μL of DNA, with template concentration below the detection limit of 10 pg/ μL (fluorometric DNA quantification assay: Qubit 1X dsDNA HS, Qiagen, Hilden, Germany), 10 μL of SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) and end concentrations of primers and probe of 0.4 μM and 0.8 μM , respectively. The following program was used: 2 min at 50°C and 10 min at 96°C, followed by 40 cycles with 25 s at 96°C for denaturation and 6 min at 55°C for annealing and extension. qPCR was carried out in duplicates on a C1000 Touch Thermal Cycler (CFX96 Real-Time System, Bio-Rad Laboratories GmbH). Absolute copy number quantification was performed by comparing cycle threshold values (c_t values) to dsDNA-standards from *Bacillus alkaliazotrophicus* strain MS 6 included in every qPCR run. Final 16S rRNA gene copy numbers per water droplet were calculated as the product of droplet volume in μL and the mean of the duplicate copy number concentrations in copy number per μL .

18S rRNA gene amplicon sequencing

18S rRNA gene amplicon sequencing was performed to investigate whether water droplets are inhabited by eukaryotic microorganisms. To this end, eight water droplets that were also subjected to 16S rRNA gene amplicon sequencing, nine puddle water samples and 68 additional water droplets that were smaller than 2 μL , and thus too small for additional ion analysis, were screened for protistan DNA. In total, 1 μL of each water droplet was used for DNA extraction as described above. DNA from puddle water was extracted with the DNeasyPowerSoil Kit (Qiagen) according to manufacturer's instructions. To circumvent the low DNA content, DNA of all droplets was pooled before performing amplicon PCR. The PCR protocol is described by (Stoeck et al. 2010) with 1 μL template DNA, 0.6 μM primers for the ribosomal V9 region 1391F and EukB, 0.5 μL HotStar HiFidelity (HiFi) polymerase (Qiagen), 5 μL 5x HotStar Buffer, but none of the supplied Q reagent. The reaction volume was adjusted to 25 μL by adding PCR-clean water (Qiagen). PCR was performed with 30 cycles. PCR products were quality checked by a 1% agarose gel electrophoresis, with positive controls and negative controls applied at every working step.

Ion chromatography

Ion chromatography was performed using the water droplet volume that remained after removing 1 μL for DNA-extraction. For comparison, we also performed ion chromatography on puddle water collected close to the oil-sampling sites. Samples were diluted 1:300 in ultrapure water (18.2 M Ωcm , TOC < 5 ppb; Millipore, Germany) and analysed with a Dionex Aquion ion chromatography system (Thermo Scientific, MA, USA) in technical

duplicates. The Aquion system was equipped with a C18 guard column (Dionex Ion Pac AG23-4 μm RFIC 2 \times 50 mm, Thermo Scientific, MA, USA). Cations were separated with the respective column (Dionex Ion Pac CS12A RFIC 2 \times 250 mm, Thermo Scientific, MA, USA) with 0.02 M methansulfonic acid as eluent. Anions were separated with the analytical column (Dionex Ion Pac AS23-4 μm RFIC 2 \times 250 mm, Thermo Scientific, MA, USA) and 0.8 mM NaHCO₃ and 4.5 mM Na₂CO₃ as eluent. The ion conductivities were finally detected by a microprocessor-controlled digital signal processor with 8 kHz square wave. Chromeleon software (Version 7.2 SR5, Thermo Scientific, MA, USA) was used for peak analysis. The technical error was assessed from known standard solutions and was below 2.4% for ion concentrations smaller than 25 μM and below 0.2% for concentrations above 1000 μM . Raw data were further processed and statistically analysed using R (R Core Team 2017). We hereafter denote the measured chloride concentrations as salinity, since the salinity of brackish and seawater mainly arises from the concentration of sodium chloride (Remane 1934). We classified the water droplets into four different salinity categories: freshwater 0–15.6 mM chloride, low brackish water 15.7–50 mM chloride, brackish water 50.1–156.1 mM chloride and brackish-marine water > 156.2 mM chloride (Remane 1934; Herlemann et al. 2011; Reineke and Schlömann 2020).

Statistical analysis

Statistical analyses were performed with vegan R package and a published R script (Torondel et al. 2016). Ion concentrations below the detection limits were set to zero (Table S2, Supporting Information; Helsel 2004). Furthermore, we only considered ions which were detected in at least 50% of the droplets as too many zeros can affect the results of statistical tests because the data set moves away from normal distribution. Ion concentrations were log-transformed after adding a constant of 1 to all data. We first tested whether ion concentrations differed between sampling sites by multivariate analysis of variance (MANOVA), followed by separate one-way analysis of variance (ANOVA) for each ion and by Tukey post-hoc tests (TukeyHSD) for pairwise comparisons of sampling sites. Permutational analysis of variance (PERMANOVA) was applied for testing if microbial communities were determined by the sampling location. Additional pairwise PERMANOVAs with 999 permutations were performed with the R package pairwise Adonis (Martinez Arbizu 2020) to identify, which sampling sites differed from each other. Effects of the ion concentrations on microbial community composition was tested by canonical correspondence analysis (CCA) against a distance matrix based on Bray–Curtis dissimilarity of community data. The relationship between chloride or sulfate and the relative abundance of individual OTUs were tested using Spearman's rank correlation with Benjamini–Hochberg multiple testing correction.

Determination of the salinity threshold

To identify the threshold value of salinity, we successively removed one data point after the other from the data set, starting with the lowest salinity and proceeding in ascending order. At each step, we tested the effect of salinity on microbial community composition using PERMANOVA. We identified the lower limit of possible threshold values of salinity as the value above which the effect on community composition became non-significant. Likewise, we successively removed individual data points from the data set by starting with the highest salinity

and proceeding in descending order. We identified the upper limit of possible threshold values for salinity as the value below which the effect became non-significant. The two obtained values described the range of possible threshold values for salinity.

RESULTS

Geochemical composition of water droplets

To study the importance of formation water geochemistry on autochthonous microbial communities entrapped in heavy oil, we measured the concentrations of 13 inorganic anions and cations of 43 water droplets that we isolated from the oil sampled at six different oil seepages on the Pitch Lake. For comparison, we also analysed the ion concentrations in puddle water that was found close to the sampling sites. Only seven ions, namely bromide, chloride, lithium, phosphate, potassium, sodium and sulfate showed sufficiently high concentrations in more than 50% of the water droplets to allow further statistical investigation. Ions such as fluoride, magnesium, nitrate, nitrite, ammonia and calcium were only detectable in a few water droplets and were not further analysed (Table S2, Supporting Information). Bromide and phosphate concentrations were relatively stable across all water droplets with a mean of 1.20 mM and 1.22 mM, respectively, but were below the detection limit of 0.7 mM and 0.9 mM in all puddle water samples (Fig. 2B; Table S2, Supporting Information). Lithium, sulfate and potassium showed a more pronounced variability between water droplets despite the small distances between individual droplets and despite sharing a common habitat (Fig. 2C–E; Table S2, Supporting Information). Nevertheless, the concentrations of these ions in water droplets obviously differed from puddle water. The concentrations of sodium and chloride strongly correlated with each other and showed the highest variability (Fig. 2F and G). The salinity of individual water droplets ranged from 5.77 mM chloride (freshwater) to 246.1 mM chloride (brackish-marine water; Fig. 2G). The salinity of puddle water was always below 11.8 mM chloride (freshwater; Fig. 2G). Thus, ion concentrations of puddle water were generally different from the ion concentrations of water droplets (Fig. 2; Table S2, Supporting Information).

Sampling site had a significant effect on the ion concentrations in the water droplets (MANOVA; Pillai's Trace = 1.4711, $F = 2.0844$, $df = 5$, $P = 0.00104$; Fig. 2A–G; Table S2, Supporting Information). Subsequent ANOVA (Table S3, Supporting Information) for each ion followed by a Tukey post-hoc test (Table S4, Supporting Information) revealed that the test results were dominated by water droplets from sampling site 6, which differed significantly from all other water droplets in particular with respect to chloride and sodium, but partially also with respect to bromide, lithium and potassium. Sulfate and phosphate concentrations did not show significant differences between sampling sites. Although the water droplets from all sampling sites spanned a wide gradient of different ion concentrations, it was remarkable that the significant differences in ion concentrations between sampling sites were mainly explained by the low values of sodium and chloride, i.e. by salinity values that were all but one in the freshwater to low brackish range.

Size and composition of microbial communities in water droplets

We estimated the size of the microbial communities in the water droplets by absolute quantification of 16S rRNA gene copy numbers using qPCR. Yet, a microbial community consists of many

different microbial species having different copy numbers of the 16S rRNA gene per cell. Additionally, each organism undergoes different PCR amplification efficiencies due to biases of the universal primers. As 16S rRNA gene copy number per cell and amplification efficiency are unknown for most species, the total copy numbers in the community measured by this qPCR-approach cannot be corrected and therefore only serve as a semi-quantitative measure of community size (Louca, Doebeli and Parfrey 2018). Results are given only for 24 out of the 43 water droplets, where both duplicate qPCR runs were successful and with mean error deviation below 10%. In these 24 droplets, absolute 16S rRNA gene copy numbers per water droplet ranged from 1.5×10^3 to 2.9×10^7 with a median of 2.6×10^5 . These results are similar to the results of Pannekens et al. (2020), who reported that individual water droplets contain on average 1.2×10^5 cells per μL . Yet, as the absolute community sizes determined by qPCR are only semi-quantitative and were not affected by sampling site or the concentrations of the inorganic ions, we did not use them further to calculate absolute species abundances in the subsequent description of microbial community composition.

The compositions of all 43 microbial communities were determined as relative abundances of the different 16S rRNA genes. The majority of OTUs was related to known inhabitants of anoxic oil reservoirs or members of phyla observed in hydrocarbon-rich environments (Li et al. 2017). The 50 most abundant OTUs represented on average $89 \pm 6\%$ (median \pm median absolute deviation) of the total abundance and contained members of *Deferritbacteraceae*, *Soehngenia*, *Tepidiphilus*, *Petrogaceae*, *Porphyromonadaceae*, *Candidatus Atribacteria*, *Thermovirga*, *Anaerobaculum*, *Parcubacteria*, *Woesearchaeota*, *Extensimonas* and *Anaerolinaceae*, an unclassified Bacterium, *Desulfobacteraceae*, *Methanotherox*, *Comamonadaceae*, *Aminicentances*, *Dehalococcoidia*, *Smithella*, *Clostridium sensu stricto*, *Calditerrivibrio* and *Acetothermia*, (see Table S5, Supporting Information). OTU richness per water droplet was highly variable, ranging from 91 (droplet 1.5) to 319 (droplet 6.37; Fig. 2H). The ten most abundant OTUs of each droplet constituted on average $77 \pm 13\%$ of the total community, which indicates rather uneven distributions dominated by only a few OTUs. These uneven distributions are supported by the moderate alpha-diversities represented by Shannon–Wiener index (H), which is mainly influenced by species richness, and by the rather high Simpson's indices of diversity (1-D), which is more influenced by community evenness and common species by putting more weight to the more abundant species in a sample. Shannon–Wiener indices ranged from $H = 1.5$ –4.8, while Simpson's indices of diversity ranged from 1-D = 0.61–0.99. High values of H and 1-D represent high sample diversity, whereby 1-D ranges between 0 and 1. The Shannon–Wiener index was significantly affected by sampling site as tested by ANOVA ($F(5,37) = 20.23$, $P < 0.001$). Yet, Tukey's post-hoc test revealed that significant differences in the Shannon–Wiener index occurred only between sampling site 6 and all other sites ($P = 0.004$), whereby droplets from site 6 harbored the most diverse and even communities ($H = 4.5 \pm 0.6$ and 1-D = 0.95 ± 0.07). Microbial community composition was strongly associated with sampling site (PERMANOVA: $df = 5$, $\text{SumsOfSqs} = 6.0407$, $\text{pseudoF} = 8.227$, $r^2 = 0.52646$, $P = 0.001$). Pairwise permutational multivariate analyses of variance revealed significant differences between microbial community compositions of each sampling site except between sites 1 and 2 and between sites 2 and 5 (Table S6, Supporting Information). These pairs of sites were the spatially closest sites to each other (Figure S1, Supporting Information).

Effect of water droplet geochemistry on microbial community composition

We analysed the relationship between the water geochemistry and microbial community composition in the water droplets using multivariate statistics and Spearman correlations. CCA of all ions revealed that chloride, sulfate and potassium had significant effects on microbial community composition ($P < 0.05$; chloride: $df = 1$, SumsOfSqs = 1.1383, pseudo $F = 4.9745$, $r^2 = 0.099$, $P = 0.001$; sulfate: $df = 1$, SumsOfSqs = 0.6370, pseudo $F = 2.7839$, $r^2 = 0.056$, $P = 0.009$; potassium: $df = 1$, SumsOfSqs = 0.5615, pseudo $F = 2.4537$, $r^2 = 0.049$, $P = 0.012$). These three factors explained 20.4% of the variability in microbial community composition (Fig. 3). To identify which of the OTUs were influenced by these factors, we investigated the correlations between single OTUs and chloride or sulfate using Spearman correlation with Benjamini–Hochberg multiple testing correction. We did not test for an association with potassium because potassium correlates with chloride (ANOVA($F(1,41) = 177.9$, $P = 2 \times 10^{-16}$). Out of all 581 OTUs, 219 showed a significant negative and 33 a significant positive correlation with chloride (Table S7, Supporting Information). 21 OTUs correlated negatively with sulfate. Out of the 273 OTUs showing significant responses, 19 belonged to the 50 most abundant OTUs found across all water droplets, which indicates that both, common as well as rare OTUs were affected by salinity and sulfate (Fig. 4; Figure S2 and Table S7, Supporting Information).

Although water droplets exhibited a wide range of ion concentrations, the significant effect of sampling site on ion concentrations was mainly explained by water droplets of sampling site 6 with particularly low brackish salinity (Figure S4, Supporting Information). This result indicated that salinity leads to changes in community composition when exceeding a certain threshold rather than in a gradual manner. To identify a potential threshold value for salinity, we investigated whether the significant effect of salinity on community composition disappears when all water droplets with salinities below a certain value are removed from the data set. Stepwise deletion of data points in ascending order of salinity revealed that the significant effect of salinity on community composition as tested by PERMANOVA disappears at a concentration of 51.4 mM chloride (Table S8, Supporting Information). Likewise, stepwise deletion of data points in descending order of salinity revealed that the significant effect of salinity on community composition disappears at a concentration of 57.3 mM chloride (Table S8, Supporting Information). The two values mark the lower and upper limit of a possible threshold value for salinity. Moreover, in the reduced data set all significant correlations between individual OTUs and chloride concentration disappeared while 10 of the significant correlations between individual OTUs and sulfate remained (Fig. 4; Table S7, Supporting Information). Together, these results indicate that salinity does not influence community composition in a gradual manner but provokes a sudden shift in community composition when exceeding a low brackish salinity threshold between 51.4 and 57.3 mM chloride. Similarly, there was a threshold-regulated effect of salinity on Shannon–Wiener diversity, which was significant in the full data set (ANOVA: ($F(1,41) = 5.374$, $P = 0.00994$), but not when water droplets below the salinity threshold were excluded (ANOVA: ($F(1,32) = 0.042$, $P = 0.773$).

Protists in water droplets

From 76 water droplets that were additionally extracted from oil, no eukaryotic 18S rRNA genes could be amplified with widely

used, general eukaryotic primers. Thus, no microbial eukaryotes were detected in the water droplets. By contrast, eukaryotic DNA could be amplified from the puddle water and from positive controls, validating the amplification protocol.

DISCUSSION

The microbial communities in the water droplets isolated from oil seepages on the Pitch Lake present a unique opportunity to investigate indigenous microbial communities from oil reservoirs. Meckenstock et al. (2014) demonstrated that the stable isotopes of hydrogen and oxygen of the droplet water were markedly different from rainwater or adjacent seawater, indicating that the water droplets and their respective microbial communities originate from the deep oil reservoir (Meckenstock et al. 2014). This study provides further support for this interpretation as it shows that the inorganic ion concentrations in the water droplets differ from the ion concentrations of puddle water collected on the Pitch Lake. The salinity of the water droplets also differed clearly from the salinity of the adjacent seawater, which contained 1100 mM chloride. Moreover, this study also shows that the water droplets, even when separated by only a few millimeters, often had markedly different concentrations of inorganic ions and different microbial communities. Thus, the water droplets harbor indigenous oil microbial communities and they represent physically isolated ecosystems that do not mix.

Indigenous microbial communities in water droplets

With the exception of *Candidatus Atribacteria* and *Tepidiphilus*, all of the identified taxa of the 50 most abundant OTUs have representatives isolated from oil fields and are described as neutrophilic, mesophilic to thermophilic, mostly anaerobic taxa, which is consistent with the moderate pH of 7.5 and the temperatures between 37.1 and 58.3 of the Pitch Lake (see Table S1, Supporting Information). The genetic potential for hydrocarbon degradation by *Candidatus Atribacteria* has been shown by genome-inferred analysis (Nobu et al. 2016; Liu et al. 2019). Only one isolate of *Candidatus Atribacteria Atribacter laminatus* gen. nov. sp. nov. (RT761) exists so far, which has been isolated from a deep sedimentary, natural gas bearing saline aquifer in Japan (Katayama et al. 2020). It was enriched anaerobically in saline mineral medium and grew best under syntrophic interactions with methanogens (Katayama et al. 2020). The genus *Tepidiphilus* has so far been described as aerobic, although one strain has been isolated from production water of an oil reservoir (Wang et al. 2020; Zhang et al. 2020). Yet, 16S rRNA gene amplicon sequencing of some anaerobic enrichment cultures from Pitch Lake oil prepared in our lab provided evidence for anaerobic growth of a strain assigned as *Tepidiphilus* (supplementary text and Table S9, Supporting Information). Also the genus *Extensimonas* from the family of *Comamonadaceae* has been described as an obligate aerobic microorganism (Zhang et al. 2013), but other members of the *Comamonadaceae* have been found in oil fields or oil polluted environments (Willems 2014). The taxa identified in this study are largely consistent with Pannekens et al. (2020), who investigated the microbial communities in water droplets from the Pitch Lake and two other natural heavy oil seeps, in the La Brea Tar Pits and the McKittrick oil field (both from California, USA). Interestingly, common inhabitants of oil reservoirs with secondary oil production such as *Pseudomonas*, *Erythrobacter*, *Aquabacterium*, *Marinobacter* or *Desulfovibrio* were not identified in Pitch Lake water droplets. This suggests that these taxa are likely exogenous contaminants, which is also supported by the fact that they do not grow at *in situ* temperatures (Magot 2005).

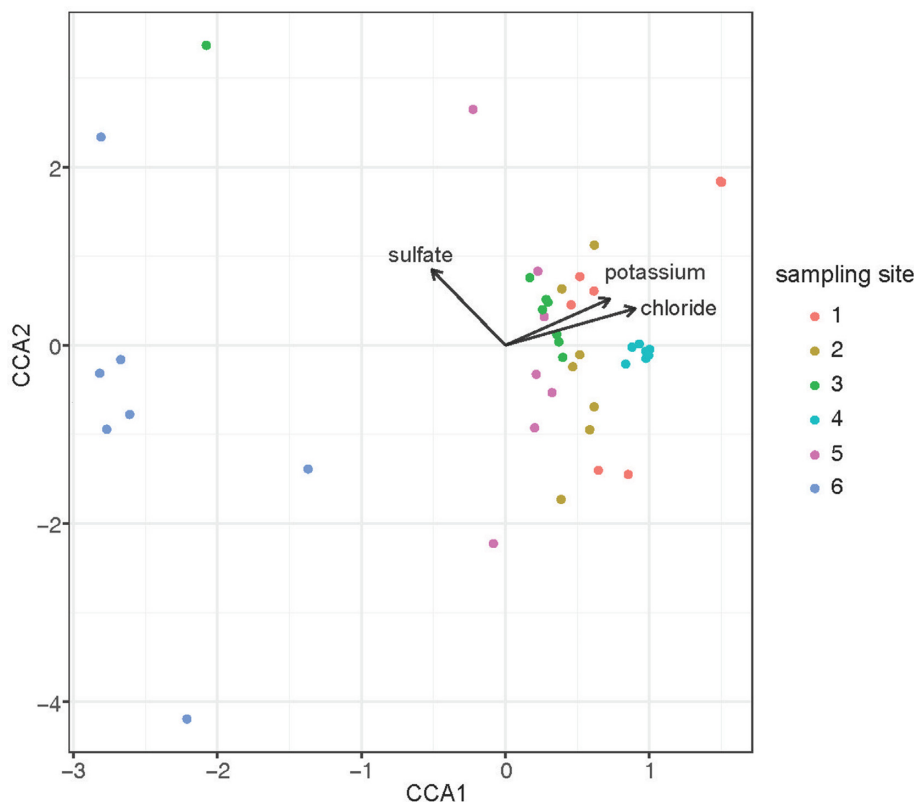


Figure 3. CCA of microbial community compositions based on Bray–Curtis dissimilarity matrix. Chloride, potassium and sulfate appear as best explanatory variables, which together explain 20.4% of the variation in the data. Note that arrows indicating chloride and potassium effects are laying on top of each other.

Overall, the taxa identified in this study demonstrated that the water droplets communities are similar to indigenous microorganisms from oil reservoirs.

Effect of salinity on microbial community composition

Salinity has been shown to be one of the most important environmental factors influencing microbial community composition in different environments worldwide (Lozupone and Knight 2007). Several studies on microbial communities from the Baltic Sea, Antarctic lakes as well as coastal estuarine wetland soils have shown that communities clustered according to salinities ranging from freshwater to hypersaline water, and that salinity can lead to drastic changes in microbial community composition and metabolism (Herlemann et al. 2011; Logares et al. 2013; Dupont et al. 2014; Zhang et al. 2021). The results of our study are in agreement with this general view and provide mechanistic insights into the response of oil communities to salinity. In contrast to previous studies, the Pitch Lake water droplets allow the analysis of a large number of individual oil microbial communities from highly similar environments because they are exposed to the same oil matrix. The microbial communities in the water droplets showed a strong shift to less diverse and uneven compositions when exceeding a salinity threshold between 51.4 and 57.3 mM chloride, i.e. at the transition from low brackish to brackish water. As the absolute community size determined by qPCR remained unaffected by this salinity threshold, we can assume that the detected changes in microbial community composition are not an indirect consequence of a salinity threshold effect on the absolute biomass. Such a drastic effect of salinity was also observed along an estuarine salinity gradient in

the Choptank River Estuary (Maryland), where increasing salinity was associated with a sudden decrease in bacterioplankton growth and single-cell activity (del Giorgio and Bouvier 2002). Rath et al. (2019) demonstrated experimentally that microbial communities of soil became less diverse and more similar to each other with increasing salinity. In contrast, Zhang et al. (2020) observed lower microbial richness and diversity in freshwater coastal estuarine wetland soils compared to the higher saline marine intertidal and supratidal zone soils, which indicates that the exact response towards salinity may depend on habitat structure.

The observed loss in diversity in the Pitch Lake water droplets at higher salinities is also consistent with other observations in oil microbial communities. Röling, Head and Larter (2003) found that the number of cultivable microorganisms in oil fields seemed to decrease with increasing oil field salinity. Furthermore, several studies on produced oil reservoirs confirmed the important role of salinity on growth, activity, relative abundance and the oil degradation potential of members of Firmicutes, Proteobacteria, Euryarchaeota and Thermotogae. However, those studies did not report if the observed salinity effect on community is related to a specific threshold level or follows a gradual pattern of change in community with gradual change in salinity (Lenchi et al. 2013; Gao et al. 2016; Li et al. 2017; Vigneron et al. 2017; Pannekens et al. 2019).

Salinity, the relative extent of biodegradation and spatial location were also identified as major drivers of microbial diversity in a comparison of 22 produced oil reservoirs in north central Louisiana, USA (Shelton et al. 2016). A total of five of these reservoirs were highly biodegraded and shallower and lower in salinity compared to all other reservoirs. Comparable to our

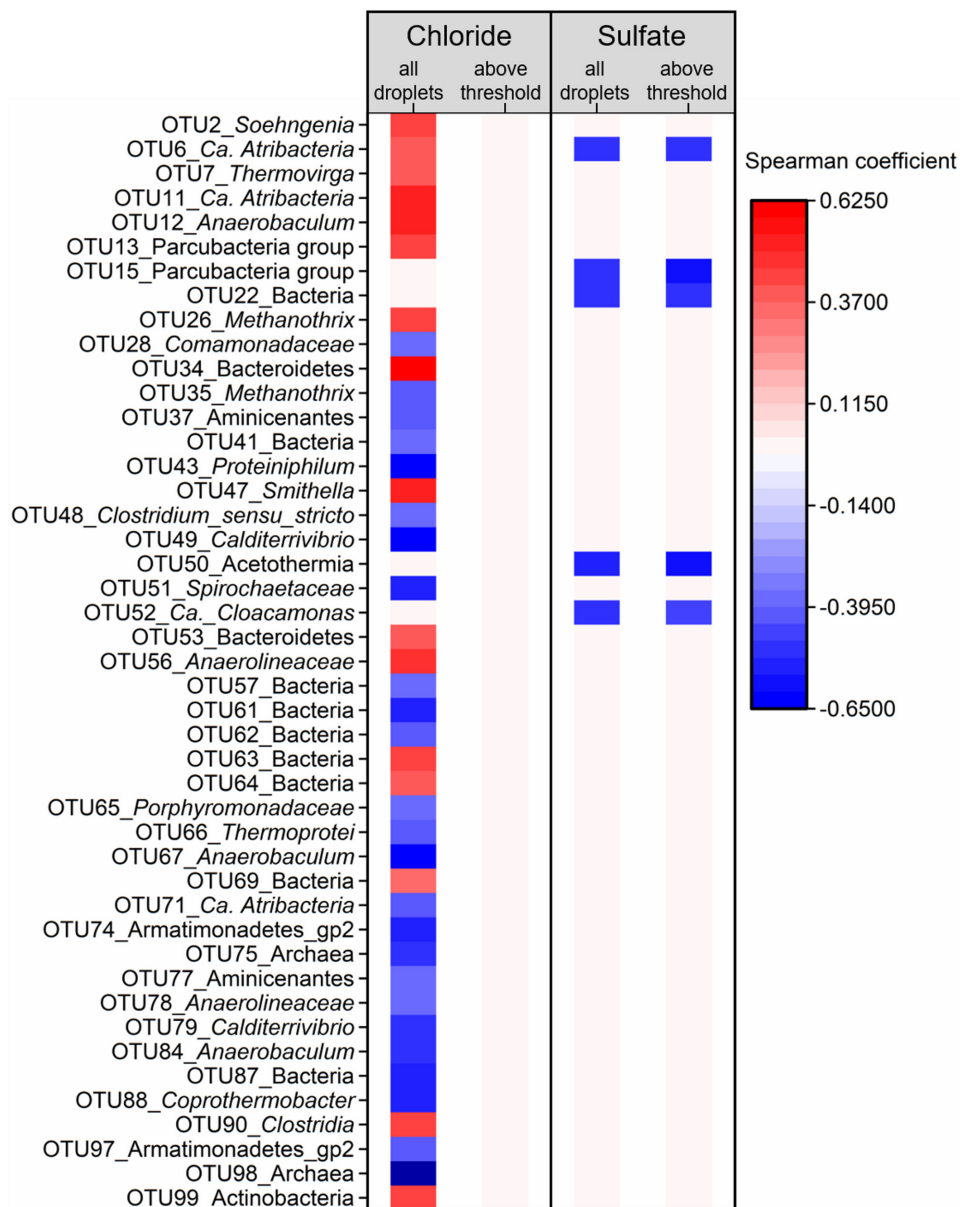


Figure 4. Spearman correlation coefficients with Benjamin-Hochberg multiple testing correction of 45 individual OTUs versus chloride and sulfate. Correlations were performed on the complete data set (left column) and one a reduced data set only containing water droplets with salinities above the threshold of 57.3 mM chloride (right column). Only coefficients with significant P-values ($P < 0.05$) are displayed (see also Table S6, Supporting Information).

study, Shelton et al. (2016) found that microbial communities switched to lower OTU richness and were unaffected by salinity once it exceeded the proposed threshold in the high salinity range between 800 and 1000 mM chloride. Interestingly, a salinity effect on oil degradation ability and methanogenesis remained detectable also above the threshold but methanogenesis seemed to be more halotolerant. Together, the results of Shelton et al. (2016) and the results of our study suggest that oil microbial communities respond to salinity at two distinct thresholds, i.e. when exceeding a lower threshold between 51.4 and 57.3 mM chloride and when exceeding a higher threshold between 800 and 1000 mM chloride.

The finding of a salinity threshold improves our understanding of how salinity shapes the composition of oil microbial communities. A salinity threshold may also have implications for future research and the management and prediction of oil

microbial communities. Furthermore, salinity threshold effects point out the heterogeneity of microbial communities in reservoirs and that a single sample might not be representative for the total reservoir.

Effects of sampling site and other geochemical parameters

The finding that the microbial communities of the water droplets are more similar within same sites than between sites, i.e. between oil seepages, may indicate that sampling sites reflect different origins of oil, water and microorganisms from within the same oil reservoir. The low salinities in some water droplets might be interpreted as an indication that this oil seep was infiltrated from a shallower aquifer or from puddle water due to turnover movement of the asphalt (Attwooll and Broome 1954;

Ostapkowicz *et al.* 2017). Yet, all detected microorganisms in the water droplets with salinity below the proposed threshold were anaerobes, and no aerobic relatives could be identified based on their 16S rRNA gene sequences, indicating that microorganisms are indigenous and were not introduced by infiltrating water. Moreover, the effect of sampling site on community composition persisted also when water droplets with salinities below the proposed threshold of 57.3 mM were excluded from the data set, indicating that the influence of sampling site is to a certain extent independent from the influence of salinity.

Generally, salinity, nutrients and the availability of electron donors and acceptors have been considered as major environmental factors determining microbial communities in oil reservoirs (Pannekens *et al.* 2019). In our study, however, we did not find evidence for an influence of nutrients, electron donors or acceptors. The average concentrations of phosphate (2.9 ± 1.21 mM) and sulfate (2.38 ± 1.64 mM) were high enough to be non-limiting (Fig. 2; Table S2, Supporting Information). The concentrations of nitrate, nitrite and ammonium were mostly below the analytical detection limits of 0.9 mM, 1.24 mM and 1.82 mM, respectively. These relatively high detection limits were caused by the tiny volume of the water droplets, which required a 300-fold sample dilution. Thus, concentrations of the different nitrogen compounds might still have been high enough to support growth. Moreover, nitrogen is generally considered not to be growth-limiting in oil reservoirs as it is contained in heterocyclic hydrocarbons and available as dinitrogen gas to nitrogen-fixing microorganisms (Magot 2005). Also, iron is unlikely to be a limiting factor because an earlier analysis of Pitch Lake oil revealed non-growth limiting iron concentrations of 3000 and 7600 mg/kg oil (Schulze-Makuch *et al.* 2011). In our study, the small size of the water droplets prohibited reliable measurements of dissolved iron concentrations.

It is also unlikely that microorganisms were limited by the availability of electron acceptors since sulfate concentrations were generally high (Fig. 2D). Interestingly, a closer look at single OTUs revealed negative correlations between sulfate and OTU richness or relative 16S rRNA gene abundances, respectively. The negative correlations may indicate that the lower sulfate concentrations resulted from growth of sulfate-reducing organisms. Yet, it is striking that we did not find high abundances of Desulfobacterota (formerly Deltaproteobacteria; Waite *et al.* 2020), which encompass the majority of sulfate-reducing microorganisms (Muyzer and Stams 2008). The low relative abundance of Desulfobacterota is consistent with an earlier characterization of the microbial community in bulk oil of the Pitch Lake (Schulze-Makuch *et al.* 2011). In other oilfields, however, sulfate-reducing bacteria have been observed to thrive in a wide range of salinities (0–4.8 M chloride). The striking absence of sulfate-reducing microorganisms despite high sulfate concentrations in the Pitch Lake and other oil fields (Magot 2005; Vigneron *et al.* 2017) is yet to be explored.

Microbial community assembly in the water droplets

We here investigated the relationship between different environmental parameters and the composition of the microbial communities in the water droplets. Environmental parameters may influence community composition either through the process of environmental filtering, *i.e.* by sorting out organisms with improper niche ranges, or by regulating biotic interactions such as competition, predation or cross-feeding, to name few (Philippot, Griffiths and Langenheder 2021). As all OTUs from droplet communities sampled in this study are affiliated with

mesophilic to thermophilic taxa from anoxic, hydrocarbon-rich environments, there is evidence for strong environmental filtering of microorganisms that are capable to thrive in this extreme environment. The threshold effect of salinity on microbial community composition also suggests that salinity acts as an environmental filter at least for a certain number of OTUs. As the water droplets communities originate from the deep oil reservoir, the salinity effects are likely occurring also at large depth. Instead, environmental parameters such as sulfate that have a more gradual effect on certain relative OTU abundances indicate that they influence community assembly through bacterial or archaeal interactions such as competition, predation or cross-feeding. The specific types of prokaryotic interaction that might have played a role could not be identified in the framework of this study, but predation by protists could be excluded as an important community assembly process as the presence of protists in the water droplets could not be confirmed. Interestingly, we also detected an effect of sampling site on microbial community composition. This indicates that water droplets from different sampling sites originated from different source communities, which means that the microbial community composition in the water droplets is to a certain amount also affected by historic events.

CONCLUSIONS

Insights into the native microbial community of oil reservoirs are hampered by the difficulty of obtaining undisturbed samples. This study showed that water droplets dispersed in heavy oil of the Pitch Lake in Trinidad harbored indigenous oil microbial communities that originate from the deep reservoir and that thrive at very different salinities. The study proposes the concept that microbial communities in oil reservoirs show a threshold-regulated response to salinity. It furthermore highlights the importance of investigating small-scale environmental heterogeneity in environments that are not well mixed and points at potential problems that may arise when analysing bulk oil samples.

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SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://www.femsec.org/) online.

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Conflict of interest. None declared.

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