Most of the microbial degradation in oil reservoirs is believed to take place at the oil-water transition zone (OWTZ). However, a recent study indicates microbial life enclosed in µl-sized water droplets dispersed in heavy oil of the Pitch Lake in Trinidad & Tobago. This life in oil suggests that microbial degradation of oil also takes place in water pockets in the oil-bearing rock of an oil leg independent of the OWTZ. However, it is unknown if microbial life in water droplets dispersed in oil is a generic property of oil reservoirs rather than an exotic exception. Hence, we took samples from three heavy oil seeps, the Pitch Lake (Trinidad & Tobago), the La Brea Tar Pits (CA, USA) and an oil seep on the McKittrick oil field (CA, USA). All three tested oil seeps contained dispersed water droplets. Larger droplets between 1-10 µl revealed high cell densities of up to $10^9$ cells ml$^{-1}$. Tests for adenosine triphosphate (ATP) content and LIVE/DEAD staining showed that these populations consist of active and viable microbial cells with an average of 60 % membrane intact cells and ATP concentrations comparable to
other subsurface ecosystems. Microbial community analyses based on 16S rRNA gene
amplicon sequencing revealed the presence of known anaerobic oil-degrading
microorganisms. Surprisingly, the community analyses showed similarities between all three
oil seeps revealing common OTUs although the sampling sites were thousands of kilometers
apart. Our results indicate that small water inclusions are densely populated micro habitats in
heavy oil and possibly a generic trait of degraded oil reservoirs.

IMPORTANCE Most of the microbial degradation in oil reservoirs takes place at the oil-
water transition zone between the oil leg and the underlying water. However, a recent study
shows microorganisms in µl-sized water droplets dispersed in biodegraded heavy oil seeps.
Here, we show that these water droplets are densely populated microhabitats containing living
microbial communities. Since these microhabitats occurred in three separate oil seeps that are
located thousands of kilometers away from each other, we propose that water droplets
populated with microorganisms might be a generic trait of biodegraded oil reservoirs.
Furthermore, microbes in these water droplets can contribute to the degradation of the oil.

Introduction

The world’s oil reservoirs are dominated by heavy oil and bitumen since anaerobic
microorganisms have degraded the oil in the absence of molecular oxygen over geological
time scales (1, 2). However, the metabolic processes and rates of biodegradation in deep oil
reservoirs remain vague due to a lack of sufficient samples and the long geological timescales
in which the degradation takes place (3). Several studies have shown that microbial
abundance and biological degradation rates are highest at the so-called oil-water transition
zone (OWTZ), i.e. the oil-water interface between an oil leg, the oil-bearing layer of an oil
reservoir, and the underlying water leg (1). With increasing distance from this transition zone,
biodegradation should be limited by lack of water, electron acceptors and dissolved inorganic nutrients such as sulfate, phosphorus, and nitrogen compounds. Hence, it is commonly assumed that no degradation takes place within the oil leg itself (1, 4). However, indicators for microbial life are found in almost all oil and water samples from reservoirs and even in heavy oil or asphalt seeps with temperatures up to 82 °C (5-14). This includes the largest natural asphalt lake, the “Pitch Lake” located on the island of Trinidad, Trinidad and Tobago.

In this natural oil seep, Meckenstock et al., (2014) discovered complex microbial communities inhabiting tiny water droplets of 1-3 µl in volume, suspended in the oil phase, in the following termed as water droplets or droplets (15). Since geochemical and isotopic analysis of the droplet water revealed a deep subsurface origin, it was concluded that the water inclusions, containing indigenous microbiota, directly ascended from the oil reservoir. In fact, water wet oil reservoirs contain water either as thin water films covering the sand grains and rock matrix or in water-filled pockets (11). Analysis of the 16S rRNA genes from single water droplets identified, among others, typical oil-degrading bacteria such as Bacteroidales, Rhodospirillales, and Sphingomonadales as well as methanogenic archaea indicating hydrogenotrophic methanogenesis as terminal electron-accepting process (15, 16).

The microbial activity in the water droplets indicated that biodegradation in oil reservoirs is not only restricted to the oil-water transition zone. Furthermore, the biodegradation might take place directly within the oil leg resulting in an increasing oil-water interface and potentially bigger overall oil degradation. These water droplets provide a unique opportunity to get insights into microbial life and degradation processes in the deep subsurface of oil reservoirs.

Nevertheless, it remained unclear if such microbial communities entrapped in water droplets are a generic feature of oil reservoirs or only a single observation from the Pitch Lake in Trinidad and Tobago. Hence, we sampled two additional natural oil seeps and studied the microbial composition of single water droplets. Furthermore, we elucidated basic features of these microbial communities including the cell density, live/dead rates of single cells,
metabolic activity, whether the microbes are living planktonic in the droplet lumen or
arranged in biofilms at the oil-water interface of each droplet, and finally the microbial
community composition as a tool to identify typical oil-degrading microorganisms.

Material and Methods

Oil sampling

Natural asphalt and heavy oil (14, 17-19) (hereafter referred as oil) was sampled from the
Pitch Lake (N 10°14’0.6882’’W 61°37’44.5638’’) on the island of Trinidad, Trinidad and
Tobago, the La Brea Tar Pits (34°03’49.7”N 118°21’25.1”W) in Los Angeles, California,
USA, and an unnamed oil seep (35°17’35.2”N 119°38’10.5”W) on the McKittrick oil field,
California, USA. The distance is 180 km between La Brea and McKittrick, 6322 km between
La Brea and Pitch Lake, and 6462 km between McKittrick and Pitch Lake. Oil surface
temperatures during sampling were 36 °C at Pitch Lake, 20.5 °C at La Brea, and 20.5 °C at
McKittrick. All spots are natural oil seeps where heavily degraded oil reaches the surface.

Oil was sampled from different spots on each particular oil seep (6 spots at Pitch Lake, 1 spot
at McKittrick, and 3 spots at La Brea). Samples were taken with 50 ml syringes, where the tip
of the syringe was cut off with a scalpel, and transferred into separate sterile glass jars (63
tales at Pitch Lake, 30 samples at McKittrick, and 12 samples at La Brea), flushed on site
directly after sampling with N₂ (5.0 grade) (Pitch Lake: Massy Gas Products, Savonetta
Estate, Trinidad & Tobago; La Brea and McKittrick: Tym Inc, Los Angeles, CA, USA), and
closed with a sterile and gastight steal lid. The jars were shipped to the laboratory by
airfreight and stored at 4 °C until further use.

Droplet in situ observations
For visualization of cells in water droplets, the oil containing the microhabitats was transferred to hanging drop slides (Brand®, Wertheim, Germany) using spatulas. The cavities of the slides were used as reservoirs to avoid compression of the oil during microscopy. Cells were stained with 2 µl of a Syto™ 9 solution (10 µM; Molecular Probes, Eugene, USA) by pipetting directly into visible water droplets, thereby the original droplet volume was increased. After injection, the samples were covered with a cover slide and incubated in the dark for 20 min. A confocal laser scanning microscope (TCS SP8 HCS A, Leica Microsystems) equipped with a 488 nm argon laser and a HC PL APO 63x 1.4 CS2 oil objective was used for visualizing the cells. Images of the Syto™ 9 stained cells were taken with an excitation wavelength of 488 nm and an emission range from 507 to 550 nm. LAS.X (version 3.5.2) and ImageJ (version 1.52i) software with Bio-Formats plug-in (version 5.8.2) were used for data processing.

**Droplet sampling**

For droplet extraction, oil samples were heated for ~30 min at 45 °C to render the oil more liquid and to allow the lighter water droplets to ascend to the sample surface. Since, the average oil temperature of the sampling spots was about 31 °C and in some cases up to 43.9 °C a cell damage due to heating deemed unlikely. Subsequently, oil samples were cooled to room temperature and water droplets were collected from the sample surface with 10 µl pipettes.

**Cell counting in individual droplets**

For cell counting 1 µl of each water droplet was diluted in 39 µl of water (18.2 MΩcm; Milli-Q® Advantage A10 device equipped with a Q-Gard®T2 filter, a Quantum®TEX filter, and a Millipak®Express 40 0.22 µm filter, Merck Millipore, Germany). Cells were counted with a light microscope (DMLS, Leica, Germany) equipped with a 40x/0.65 ocular (C Plan, Leica,
Germany) and with a counting chamber (Thoma, Brand GmbH + Co KG, Germany). In total, 122 droplets of each oil seep were examined.

To validate the first counting, additional 12 droplets from Pitch Lake were stained with 4’,6-diamidino-2-phenylindole (DAPI). To this end, 1 µl of each water droplet sampled from Pitch Lake oil was mixed with 1 ml of DAPI solution (25 µg ml⁻¹; Sigma, Steinheim, Germany), incubated for 20 min in the dark, and subsequently filtered through 0.2 µm polycarbonate membrane filters (EMD Millipore Isopore™, Cork, Ireland). Filters were stored at 4 °C until further use. Cells were counted with an epifluorescence microscope (Axio scope.A1; Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with a 100x/1.25 oil objective (N-Achroplan; Carl Zeiss Microscopy GmbH, Göttingen, Germany).

**Determination of cell membrane integrity in individual droplets**

Membrane integrity of cells isolated from water droplets was investigated with the Live/Dead™ BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, USA). The membrane permeability of propidium iodide can be increased by too high propidium iodide concentrations or other influences such as oxygen, heat, or cells in their division cycle, leading to an overestimation of membrane-damaged cells (20-23). To avoid false negative staining results due to overstaining with propidium iodide different propidium iodide concentrations were tested by staining an unpublished sulfate-reducing enrichment culture from the Pitch Lake. The manufacturer’s instructions were modified according to the test results (not shown) and the staining reagent concentrations were adjusted to 1.65 mM Syto™9 and 0.05 mM propidium iodide, respectively.

Isolated droplets from Pitch Lake (PL), McKittrick (MC), and La Brea Tar Pits (LB) were diluted in 1 ml of substrate free, fresh water medium (24) (for PL) or phosphate-buffered saline (pH 7.5) (for MC and LB), respectively. Then, 3 µl of staining reagent were added to
each droplet, followed by incubation for 20 min at room temperature in the dark. For dead controls, approx. 15 µl droplet water were pooled and 2x2 µl of the mixture were diluted each in 1 ml 70 % isopropanol (BioReagent for molecular biology; Sigma-Aldrich, St. Louis, USA). The controls were incubated for 1-2 h at 60 °C and 900 rpm in a thermoshaker (ThermoMixer X, Eppendorf AG, Hamburg, Germany). Afterwards, all samples were filtered through 0.2 µm polycarbonate membrane filters (EMD Millipore Isopore™, Cork, Ireland). The filters were stored at 4 °C in the dark. Two confocal laser scanning microscopes were used for visualizing microorganisms in the water droplets. The Axiovert 100 M microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) was equipped with a 100x/1.3 Plan-NeoFluar oil objective, LP 385 and LP 650 filters, an argon laser 488 nm, and the software LSM 510; the TCS SP8 HCS A microscope (Leica Microsystems, Germany), was equipped with a HC PL APO 63x 1.4 CS2 oil objective, a 488 nm and 514 nm argon laser, and the software LAS.X (version 3.5.2)]. Images of the Syto™9 stained cells were taken with an excitation wavelength of 488 nm and an emission range from 507 to 550 nm. Images of propidium iodide were taken with an excitation wavelength of 514 nm and an emission range from 617 to 680 nm. ImageJ (version 1.52i) software with Bio-Formats Plugin (version 5.8.2) was used for analysis.

**ATP quantification**

Adenosine triphosphate (ATP) in the isolated water droplets was quantified with the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, USA) according to the manufacturer’s instructions. From each isolated droplet, 3 µl were diluted in 97 µl water and mixed with 100 µl BacTiter-Glo™ Reagent. After 5 min incubation, all samples were measured with a luminometer (Glomax 20/20 Luminometer, Promega, Sunnyvale, USA). To exclude possible matrix effects during measurements of hydrocarbon-rich water, inhibition tests were performed with matrix water. To this end, oil was heated to 80 °C, transferred into
50 ml centrifuge tubes and subsequently centrifuged for 2 h at 3,214 x g (centrifuge 5810 R; Eppendorf, Hamburg, Germany). After centrifugation, approximately 200 µl water could be extracted from the ~60 ml of oil and mixed with 200 µl of BacTiter-Glo™ reagent (Promega, Madison, USA). The solution was incubated overnight for full ATP removal. For luciferase inactivation, the mixture was heated 2 × 10 min in a thermoshaker (ThermoMixer X, Eppendorf AG, Hamburg, Germany) at 95 °C and 900 rpm. Afterwards, the ATP and luciferase free matrix water was diluted with water (18.2 MΩcm, final concentration 3 % v/v, equivalent to sample volume). The processed matrix water served as solvent for 10 mM ATP (Promega, Madison, USA) used as reference.

**DNA extraction, 16S rRNA gene amplification, library preparation and sequencing**

We developed a protocol consisting of two lysis steps for the extraction of DNA from tiny water droplets with a volume as small as 1 µl. In order to lyse gram-positive bacteria, 1 µl of an enzyme cocktail was mixed with 1 µl of droplet water and incubated for 1 h at 37 °C. The enzyme cocktail consisted of 2.5 U µl⁻¹ lysozyme (Sigma-Aldrich, USA), 0.6 U µl⁻¹ mutanolysin (Sigma-Aldrich, USA), and 0.048 U µl⁻¹ lysostaphin (Sigma-Aldrich, USA) and was designed to achieve an unbiased representation of the microbial community based on the results of (25). In order to lyse gram-negative bacteria and archaea, 2 µl of alkaline solution were added and the mixture was incubated for 5 min at room temperature. The alkaline solution contained 0.4 M KOH (VWR, Darmstadt, Germany) and 0.1 M dithiothreitol (Sigma-Aldrich, USA) (26). Alkaline lysis was stopped by adding 2 µl Tris-HCl (pH 4) (Fisher Scientific, Schwerte, Germany).

Amplification of the 16S rRNA genes, library preparation and sequencing were performed on two technical replicates per DNA sample. The 16S rRNA gene library preparation was accomplished according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Part # 15044223 Rev. B) with the following modifications. 16S rRNA gene sequences
were amplified by targeting the hypervariable V3-V4 region with forward primer Pro341f (5' -
CCT ACG GGN BGC ASC A-3') and overhang adaptor (5' -TCG TCG GCA GCG TCA
GAT GTG TAT AAG AGA CAG CCT ACG GGN BGC ASC A-3') and with reverse primer
Pro805r (5' -GAC TAC NVG GGT ATC TAA TCC
TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CNV GGG TAT CTA ATC 
C-3'). The choice of primers and hypervariable region aimed at covering the broadest possible
spectrum of both bacteria and archaea (27, 28). Amplicon PCRs were performed in reaction
volumes of 25 µl, each containing 2 µl of extracted DNA, 12.5 µl of 2X KAPA HiFi Hot Start
ReadyMix (KAPA Biosystems, Massachusetts, USA) and 0.25 µM of each primer with
overhang adaptor. The thermocycling protocol started with 5 min at 95 °C, followed by a
touchdown protocol with 10 cycles of 30 s at 95 °C, 30 s at 60-55 °C, with a decline of 0.5 °C
per cycle, and 30 s at 72 °C, then continuing with 30 cycles of 95 °C for 30 s, 54 °C for 30 s
and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplicon PCR products were
checked by agarose gel electrophoresis with 1 % (w/v) gels. Purification of amplicons
proceeded according to the Illumina protocol using 16 µl of MagSi-NGS Prep-Plus magnetic
beads for 20 µl of PCR product (Steinbrenner Laborsysteme GmbH, Mannheim, Germany).
Purified samples were employed as templates for Index PCRs using the Nextera XT Index kit
v2 Set D (Illumina, FC-131-2004, USA) and the following thermocycling protocol: 95 °C for
3 min, then 10 cycles with 95 °C for 30 s, 55 °C for 45 s and 72 °C for 60 s, and a final
extension at 72 °C for 5 min. Index PCR products were checked by agarose gel
electrophoresis and purified with magnetic beads as described above. DNA concentration of
each sample was quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen, USA) and
normalized to 4 ng µl⁻¹ using 10 mM Tris-Cl, pH 8.5 (Buffer EB, Qiagen, Germany). About
96 normalized samples were combined in one tube and submitted to the sequencing company
(Eurofins Genomics Germany GmbH, Germany) for sequencing on the Illumina MiSeq
platform. Sequencing reads were demultiplexed by the sequencing facility.
Bioinformatic analysis was carried out using mothur (v.1.40.5, last updated: 06/19/2018) MiSeq SOP (29, 30). After merging forward and reverse reads, sequences with ambiguous bases, shorter than 380 bp but longer than 470 bp, were removed from the data set. All remaining unique sequences were aligned to the bacterial database SILVA v132 customized to the region of interest (31-33). Chimeras and non-ribosomal sequences were removed and taxonomic classification was assigned based on RDP, trainset16 (Ribosomal Database Project) (34). Sequences were clustered into operational taxonomic units (OTUs) by defining a 97% similarity cut-off (setting of 0.03 distance limit). Reads were rarified via mothur to the lowest detected read number of 14281 of sample 46_PL. The R package phyloseq (35) was applied for diversity and community analysis of rarefied samples. OTUs with a read number below 10 and OTUs which were only abundant in one of the two technical replicates were rated as rare species or sequencing mistake and removed from the dataset. Afterwards, technical replicates were pooled by calculating the mean number of reads for each OTU. Raw sequencing reads were deposited in the NCBI database in Bioproject ID PRJNA546121.

Results

Distribution and density of microorganisms in water droplets

In order to determine the localization of microorganisms in water droplets enclosed in oil of the Pitch Lake in Trinidad we performed confocal laser scanning microscopy (CLSM), which revealed small water inclusions dispersed in the oil (Fig. 1). Pictures of Syto™9-stained specimen clearly showed microorganisms in these droplets but cells were only found in water inclusions larger than 10-20 µm diameter (Fig. 1). Due to the addition of the staining solution, the actual droplet volume of the droplets was artificially enhanced. Total cell counts of the lumen of isolated water droplets revealed that most droplets contained microbial cells with
abundances ranging from $5.6 \times 10^3$ up to $1.2 \times 10^6$ cells µl$^{-1}$ (Fig. 2). The average cell numbers ranged from $2.6 \times 10^4$ cells µl$^{-1}$ ($N = 10$) in the McKittrick water droplets, over $1.2 \times 10^5$ cells µl$^{-1}$ ($N = 10$) in the ones from the Pitch Lake, to $4.5 \times 10^5$ cells µl$^{-1}$ ($N = 10$) in La Brea Tar Pit droplets. The highest cell density in a single droplet was found in La Brea oil with $1.2 \times 10^6$ cells µl$^{-1}$. According to the fluorescence microscopy results shown above, it is likely that some cells were attached at the oil-water interface and were not detected in this counting. The observed cells differed in size, shape, and composition, indicating diverse communities inside different droplets. The most abundant morphologies were rods and diplobacilli respectively, but also cocci, diplococci, and filamentous microorganisms were observed. The epifluorescence counting of filtered droplet water (data not shown) confirmed the counting via Thoma chamber.

**Water droplets contain living cells**

To analyze if the observed cells were living microorganisms, we applied Live/Dead staining to differentiate between membrane-intact and membrane-damaged cells (Fig. 3). Membrane-intact cells (green fluorescent signal), were found in all populated droplets. The ratio between membrane-intact and membrane-damaged cells (red fluorescent signal) varied between water droplets from the three oil seeps. Nevertheless, the average of intact cells was around 53 % in all three seeps indicating that a substantial amount of the observed cells were alive (Fig. 4). In dead controls, 98 % of the cells were membrane-damaged indicating the reliability of the method (results not shown). Furthermore, we determined the ATP concentration in single droplets, which is an indicator for active and live cells. Control ATP standards dissolved in water or water extracted from Pitch Lake oil did neither indicate inhibition nor enhancement of the obtained signal (data not shown). ATP was detected in most of the tested droplets but the average ATP concentration in extracted droplets varied within and between the three oil
seeps (Fig. 5). The lowest average ATP concentration appeared in droplets from the Pitch Lake with 21.8 pM, followed by McKittrick with 194.8 pM, and La Brea with 492.2 pM.

Microbial community analysis

16S rRNA gene sequencing was used for evaluating similarities between the three oil seeps which might reveal a core community of typical oil-degrading microorganisms living in the water droplets. Bacterial and archaeal community compositions were analyzed by 16S rRNA gene sequencing of 10-12 separate water droplets from each oil seep. After read processing, quality filtering, and rarifying every sample to 14,281 reads, 4.6 million sequences were recovered across all samples. Sequences were clustered into 558 OTUs at a 97% sequence similarity cut-off. Among those, 525 OTUs belonged to 26 bacterial phyla and 33 OTUs to four archaeal phyla. The individual water droplets contained between 64 and 316 OTUs each.

Typical microbial inhabitants of oil reservoirs were found in all water droplets indicating that the water droplets originated from the reservoir and were not introduced from the surface of the oil seep. Most prominent representatives belonged to the bacterial phyla Proteobacteria, Bacteroidetes, Firmicutes, Synergistetes, Deferrribacteres, Thermotogae, Chloroflexi, Bacteroidia, and candidate phylum “Atribacteria”, while Euryarchaeota and Woesearchaeota represented the dominant archaeal phyla. OTUs, which could not be classified by the RDP classifier, were reclassified using Blast against the non-redundant NCBI nucleotide database (36). Most unclassified OTUs could be assigned to the candidate phyla “Atribacteria” and “Parcubacteria”.

The ten most abundant OTUs in the respective oil seep represented 8.38 %, 4.45 %, and 7.45 % of Pitch Lake, McKittrick, and La Brea overall droplet communities, respectively, indicating that the communities were not dominated extensive by individual OTUs. This is
supported by the Simpson diversity indices of $D = 0.75 \pm 0.11$ for Pitch Lake, $D = 0.94 \pm 0.02$ for McKittrick, and $D = 0.80 \pm 0.18$ for La Brea droplets, which point at rather evenly distributed, and thus relatively diverse communities. Alpha-diversities by Shannon-Wiener indices of $H = 2.2 \pm 0.5$ for Pitch Lake droplets, $H = 3.7 \pm 0.2$ for McKittrick, and $H = 2.7 \pm 1.0$ for La Brea droplets, indicate the most diverse community in McKittrick droplets. The compositional differences between the droplet communities were calculated as Bray-Curtis dissimilarities and indicate that the individual droplet community compositions were more similar within the respective oil seeps, leading to a clustering of the three seeps separate from each other (Fig. 6). Among the 558 OTUs identified in the three investigated oil seeps, 88 OTUs (16 %) were found in all three oil seeps. Furthermore, 8 out of these were present in 97-100 % of the analyzed droplets, building a significant core community. This core community covered a relative abundance of 3.18 % (Pitch Lake), 1.22 % (McKittrick), and 1.60 % (La Brea), of the droplet communities within the respective oil seeps. Furthermore, La Brea and McKittrick shared 185 OTUs (33 %), La Brea and Pitch Lake had 17 OTUs (3 %) in common, and McKittrick and Pitch Lake 11 OTUs (2 %). Even though many OTUs were present in all three oil seeps, the relative abundance of each OTU varied greatly between droplets within each oil seep. Nevertheless, the large percentage of the core community indicates a high degree of specialization. Pitch Lake droplets contained 31 unique OTUs, McKittrick 79 OTUs (14 %), and La Brea 147 OTUs (26 %), the top 10 OTUs based on a family level of each individual droplet are shown in Fig. S1.

**Discussion**

The discovery of microorganisms in tiny water droplets suspended in oil of the Pitch Lake in Trinidad and Tobago revealed a new habitat for microorganisms and a new concept for oil degradation (15). However, it was still unclear if the finding of microorganisms in water
droplets dispersed in oil was a unique observation from the Pitch Lake in Trinidad, or if life in oil is a generic feature of oil reservoirs. Therefore, we sampled three natural oil seeps located at the Pitch Lake in Trinidad, the McKittrick oil field in California, and the La Brea Tar Pits in Los Angeles, USA to look for such water droplets. Furthermore, we aimed at characterizing the principle structures of the microbial communities in this extreme environment.

Indeed, similar small water droplets of 1-10 µl size were found in all three natural oil seeps. Cell counting of the suspended microorganisms in the droplets indicated dense populations up to $1.2 \times 10^6$ cells µl$^{-1}$. This is an astonishing density compared to other deep subsurface habitats that only contain, for example, a thousand-fold less cells with around $10^5$ to $10^6$ cells cm$^{-3}$ (corresponding to $10^2$-$10^3$ cells µl$^{-1}$), depending on the depth (1). With densities of $1 \times 10^4$ to $4.25 \times 10^4$ cells ml$^{-1}$, the microbial abundance in production water from oil reservoirs is also much lower compared to our droplets (9, 37). Moreover, the micrographs of our droplets indicated that some microorganisms seemed to grow at the oil-water interface of the droplets. Biofilm formation on hydrocarbon-oil interfaces was shown earlier for microbial degradation of alkanes or polycyclic aromatic hydrocarbons (38, 39). Hence, it is likely that the microorganisms in the water droplets form biofilms at the oil-water interface, which possibly increases the bioavailability and facilitates the degradation of n-alkanes ($C_8$-$C_{28}$) and n-alcohols ($C_{12}$ and $C_{16}$) by sorption of hydrocarbons to extra polymeric substances.

The microorganisms in water droplets of all three oil seeps were not only present in high densities but also alive as indicated by a large portion of membrane-intact cells. Due to the technical limitations of the life-dead assay, the true number of intact cells was most likely higher than the estimated 60 %.

Furthermore, metabolic activity could be shown by the presence of ATP, which is a constant value for living cells because microorganisms have to sustain an energy homoeostasis (40). With approximately $1.47 \times 10^{-21}$ mol ATP per cell, the microorganisms in the water droplets contained small quantities of ATP indicating very little
activity compared to other environmental habitats that ranged from $10^{21}$ to $10^{15}$ mol ATP per cell (40-42). Since ATP is rapidly consumed in the presence of biomass, we conclude that the detected ATP stemmed from living cells (40, 42-44). Hence, the life-dead stain and the ATP determination indicate that the detected microorganism in the water droplet were alive and active and not only dead microbes that were accidentally entrapped in the droplets.

The microbial community composition showed similarities in the three tested oil seeps and the calculated low Shannon-Wiener and high Simpson diversity indices are similar to other oil field microbial communities (45-48). Such values may reflect a high specialization and a long isolation of the community, leading to reduced diversity but evenly composed microbial communities in the three sampled oil seeps. However, the low Shannon diversity is also certainly a consequence of the small sample size. It is anyway problematic to compare diversities of samples from different studies if they have not been rarified or normalized to a common size of the data set.

Although the communities in the droplets were clearly more similar within one seep as compared to the other two seeps they shared a significant number of OTUs despite the fact that they are located hundreds (La Brea and McKittrick) or thousands (La Brea, McKittrick, and Pitch Lake) of kilometers away from each other. Most of these core OTUs were shared between the La Brea and McKittrick oil seeps (33 %), which are geographically closer to each other, but La Brea or McKittrick shared also 16 % of all detected OTUs with the Pitch Lake droplets. These commonalities between the three different seeps support the paradigm of Baas Becking (1934): “everything is everywhere, but the environment selects” (49, 50), especially since oil reservoirs represent a highly selective and extreme environment. Although, in principle, core communities can also consist of microorganisms that are not essential to the habitat, e.g. when samples are exposed to strong microbial dispersal (51, 52), this possibility
is unlikely for the water droplets because they constitute highly isolated ecosystems that have probably been separated from each other over longer time scales (15).

These conclusions are supported by comparing the droplet communities to microbiomes found in other oil fields. The most abundant families from our droplets occurred in all three reservoirs and contained anaerobic or facultative anaerobic members which were also reported for other oil reservoirs all over the world at mesophilic to thermophilic conditions (5, 7, 53-76).

The finding that water droplets populated with active microbial communities are found in the three tested oil seeps is a strong indication that life in water droplets dispersed in oil could be a generic feature of oil reservoirs. Moreover, the remarkable similarities of the microbial communities in physically isolated water droplets of geographically very distant oil seeps indicates that this microbial life is highly adapted.

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Disclosure Statement

The authors report no competing financial interests.

References

1. Head IM, Jones DM, Larter SR. 2003. Biological activity in the deep subsurface and the origin of heavy oil. Nature 426:344.
2. Aitken CM, Jones DM, Larter S. 2004. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature 431:291.
3. Larter S, Wilhelms A, Head I, Koopmans M, Aplin A, Di Primio R, Zwach C, Erdmann M, Telnaes N. 2003. The controls on the composition of biodegraded oils in the deep subsurface - part 1: biodegradation rates in petroleum reservoirs. Org Geochem 34:601-613.
4. Bennett B, Adams J, Gray N, Sherry A, Oldenburg T, Huang H, Larter S, Head I. 2013. The controls on the composition of biodegraded oils in the deep subsurface – Part 3. The impact of microorganism distribution on petroleum geochemical gradients in biodegraded petroleum reservoirs. Org Geochem 56:94-105.
5. Gao PK, Tian HM, Wang YS, Li YS, Li Y, Xie JX, Zeng B, Zhou JF, Li GQ, Ma T. 2016. Spatial isolation and environmental factors drive distinct bacterial and archaeal communities in different types of petroleum reservoirs in China. Sci Rep 6.
6. Lin JZ, Hao B, Cao GZ, Wang J, Feng Y, Tan XM, Wang WD. 2014. A study on the microbial community structure in oil reservoirs developed by water flooding. J Pet Sci Eng 122:354-359.
7. Magot M, Ollivier B, Patel BKC. 2000. Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 77:103-116.
8. Pannekens M, Kroll L, Müller H, Mbow FT, Meckenstock RU. 2018. Oil reservoirs, an exceptional habitat for microorganisms. New Biotechnol 49:1-9.
9. Lenchi N, Inceoglu O, Kebbouche-Gana S, Gana ML, Lilros M, Servais P, Garcia-Armisen T. 2013. Diversity of microbial communities in production and injection waters of Algerian oilfields revealed by 16S rRNA gene amplicon 454 pyrosequencing. Plos One 8.6.
10. Li X-X, Mbadinga SM, Liu J-F, Zhou L, Yang S-Z, Gu J-D, Mu B-Z. 2017. Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. Int Biodeter Biodegr 120:170-185.
11. Youssef N, Elshahed MS, McInerney MJ. 2009. Microbial processes in oil fields: culprits, problems, and opportunities. Adv Appl Microbiology 66:141-251.
12. Bernard FP, Connan J, Magot M. 1992. Indigenous Microorganisms in connate water of many oil fields: a new tool in exploration and production techniques. In SPE Annual Technical Conference and Exhibition. Society of Petroleum Engineers.
13. Cai M, Nie Y, Chi C-Q, Tang Y-Q, Li Y, Wang X-B, Liu Z-S, Yang Y, Zhou J, Wu X-L. 2015. Crude oil as a microbial seed bank with unexpected functional potentials. Sci Rep 5:16057.

14. Kim JS, Crowley DE. 2007. Microbial diversity in natural asphalts of the rancho la brea tar pits. Appl Environ Microbiol 73:4579-4591.

15. Meckenstock RU, von Netzer F, Stumpf C, Lueders T, Himmelberg AM, Hertkorn N, Schmitt-Kopplin P, Harir M, Hosein R, Haque S. 2014. Water droplets in oil are microhabitats for microbial life. Science 345:673-676.

16. Head I, Larter S, Gray N, Adams J, Aitken C, Jones D, Rowan A, Huang H, Röling W. 2010. Hydrocarbon degradation in petroleum reservoirs, p 3097-3109, Handbook of hydrocarbon and lipid microbiology.

17. Meyer RF, Attanasi ED. 2003. Heavy oil and natural bitumen - strategic petroleum resources.

18. Hein FJ. 2013. Overview of heavy oil, seeps, and oil (tar) sands, California.

19. Schulze-Makuch D, Haque S, Antonio MRD, Ali D, Hosein R, Song YC, Yang JS, Zaikova E, Beckles DM, Guinan E, Lehto HJ, Hallam SJ. 2011. Microbial life in a liquid asphalt desert. Astrobiology 11:241-258.

20. Frösler J, Panitz C, Wingender J, Flemming H-C, Retterberg P. 2017. Survival of Deinococcus geothermalis in biofilms under desiccation and simulated space and martian conditions. Astrobiology 17:431-447.

21. Amor KB, Breeuwer P, Verbaarschot P, Rombouts FM, Akkermans AD, De Vos WM, Abee T. 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead Bifidobacterium cells during bile salt stress. Appl Environ Microbiol 68:5209-5216.

22. Davey HM, Hexley P. 2011. Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. Environ Microbiol 13:163-171.

23. Shi L, Günther S, Hübbschmann T, Wick LY, Harms H, Müller S. 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry A 71:592-598.

24. Widdel F, Pfennig N. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Archives of microbiology 129:395-400.

25. Yuan S, Cohen DB, Ravel J, Abd Z, Forney LJ. 2012. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PloS One 7:e33865.

26. He J, Du S, Tan X, Arefin A, Han CS. 2016. Improved lysis of single bacterial cells by a modified alkaline-thermal shock procedure. Biotechniques 60:129-135.

27. Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2014. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. PloS One 9:e105592.

28. Grappeuntner S, Loeper N, Künzel S, Baines JF, Rupp J. 2018. Selection of validated hypervariable regions is crucial in 16S-based microbiota studies of the female genital tract. Sci Rep 8:9678.

29. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:5537-5541.

30. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79:5112-5120.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41:D590-D596.

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO. 2013. The SILVA and “all-species living tree project (LTP)” taxonomic frameworks. Nucleic Acids Research 42:D643-D648.

Glöckner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, Bruns G, Yarza P, Peplies J, Westram R. 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol 261:169-176.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261-5267.

McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8:e61217.

Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. 2008. Database indexing for production MegaBLAST searches. Bioinformatics 24:1757-1764.

Orphan VJ, Taylor LT, Hafenbradl D, Delong EF. 2000. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. Appl Environ Microbiol 66:700-11.

Klein B, Bouriat P, Goulas P, Grimaud R. 2010. Behavior of Marinobacter hydrocarbonoclasticus SP17 cells during initiation of biofilm formation at the alkane-water interface. Biotechnol Bioeng 105:461-468.

Johnsen AR, Karlson U. 2004. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. Appl Microbiol Biotechnol 63:452-459.

Shama G, Malik DJ. 2013. The uses and abuses of rapid bioluminescence-based ATP assays. Int J Hyg Environ Health 216:115-125.

Hammes F, Goldschmidt F, Vital M, Wang Y, Egli T. 2010. Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Res 44:3915-3923.

Wilson CA, Stevenson LH, Chrzanowski TH. 1981. The contribution of bacteria to the total adenosine triphosphate extracted from the microbiota in the water of a salt-marsh creek. J Exp Mar Biol Ecol 50:183-195.

Cowen DA, Casanueva A. 2007. Stability of ATP in Antarctic mineral soils. Polar Biology 30:1599-1603.

Karl DM. 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol Rev 44:739.

Xiao M, Zhang Z-Z, Wang J-X, Zhang Q-G, Luo Y-J, Song Z-Z, Zhang J-Y. 2013. Bacterial community diversity in a low-permeability oil reservoir and its potential for enhancing oil recovery. Bioreourc Technol 147:110-116.

Xiao M, Sun S-S, Zhang Z-Z, Wang J-M, Qiu L-W, Sun H-Y, Song Z-Z, Zhang B-Y, Gao D-L, Zhang G-Q. 2016. Analysis of bacterial diversity in two oil blocks from two low-permeability reservoirs with high salinities. Sci Rep 6:19600.

Silva T, Verde L, Neto ES, Oliveira V. 2013. Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. Int Biodeter Biodegr 81:57-70.

Kobayashi H, Endo K, Sakata S, Mayumi D, Kawaguchi H, Ikarashi M, Miyagawa Y, Maeda H, Sato K. 2012. Phylogenetic diversity of microbial communities associated with the crude-oil, large-insoluble-particle and formation-water components of the...
reservoir fluid from a non-flooded high-temperature petroleum reservoir. J Biosci Bioeng 113:204-210.

49. Fondi M, Karkman A, Tamminen MV, Bosi E, Virta M, Fani R, Alm E, McNerney JO. 2016. “Every gene is everywhere but the environment selects”: Global geolocalization of gene sharing in environmental samples through network analysis. Genome Biol Evol 8:1388-1400.

50. Baas-Becking LGM. 1934. Geobiologie; of inleiding tot de milieukunde. WP Van Stockum & Zoon NV.

51. Shade A, Stopnisek N. 2019. Abundance-occupancy distributions to prioritize plant core microbiome membership. Current opinion in microbiology 49:50-58.

52. Sieber M, Pita L, Weiland-Bräuer N, Dirksen P, Wang J, Mortzfeld B, Franzenburg S, Schmitz RA, Baines JF, Fraune S. 2019. Neutrality in the Metaorganism. PLoS biology 17:e3000298.

53. Orlygsson J, Kristjansson JK. 2014. The Family Hydrogenophilaceae. The Prokaryotes, 859-868.

54. Arkell N, Kuznetsov P, Kuznetsova A, Foght JM, Siddique T. 2015. Microbial metabolism alters pore water chemistry and increases consolidation of oil sands tailings. J Environ Qual 44:144-153.

55. Okoro C, Smith S, Chiejina L, Lumactud R, An D, Park HS, Voordouw J, Lomans BP, Voordouw G. 2014. Comparison of microbial communities involved in souring and corrosion in offshore and onshore oil production facilities in Nigeria. J Ind Microbiol Biotechnol 41:665-678.

56. Manaia CM, Nogales B, Nunes OC. 2003. Tepidiphilus margaritifer gen. nov., sp. nov., isolated from a thermophilic aerobic digester. Int J Syst Evol Microbiol 53:1405-1410.

57. Sakamoto M. 2014. The family porphyromonadaceae. The prokaryotes, 811-824.

58. Grabowski A, Nercessian O, Fayolle F, Blanchet D, Jeanthon C. 2005. Microbial diversity in production waters of a low-temperature biodegraded oil reservoir. Fems Microbiol Ecol 54:427-443.

59. Willems A, Gillis M. 2005. Comamonadaceae Willems, De Ley, Gillis and Kersters 1991a, 447VP, p 686-688, Bergey's manual of systematic bacteriology, vol. 2 Springer.

60. Willems A. 2014. The family Comamonadaceae. The Prokaryotes: Alphaproteobacteria and betaproteobacteria:777-851.

61. Wen A, Fegan M, Hayward C, Chakraborty S, Sly LI. 1999. Phylogenetic relationships among members of the Comamonadaceae, and description of Delftia acidovorans (den Dooren de Jong 1926 and Tamaoka et al. 1987) gen. nov., comb. nov. Int J Syst Evol Microbiol 49:567-576.

62. Willems A, Pot B, Falsen E, Vandenme P, Gillis M, Kersters K, De Ley J. 1991. Polyphasic taxonomic study of the emended genus Comamonas: relationship to Aquaspirillum aquaticum, E. Falsen group 10, and other clinical isolates. Int J Syst Evol Microbiol 41:427-444.

63. Pérez-Pantoja D, Donoso R, Agulló L, Córdova M, Seeger M, Pieper DH, González B. 2012. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Microbiol 14:1091-1117.

64. Oren A. 2014. The family Methanotrichaceae. The Prokaryotes. The Prokaryotes, 298-306.

65. Lin J, Hao B, Cao G, Wang J, Feng Y, Tan X, Wang W. 2014. A study on the microbial community structure in oil reservoirs developed by water flooding. J Pet Sci Eng 122:354-359.
Kano S, Mukaidani T, Hattori Y, Fujiwara K, Miyagawa Y, Takabayashi K, Maeda H, Okatsu K. 2009. Diversity of indigenous anaerobes and methane conversion system from reservoir oil by indigenous anaerobes in depleted oil fields. J Jpn Petrol Inst 52:297-306.

Kuever J. 2014. The Family Desulfobulbaceae, p 75-86, The Prokaryotes.

Kuever J. 2014. The family Syntrophobacteraceae. The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria, 289-299.

Galushko A, Kuever J. 2019. Desulfoprunum. Bergey’s manual of systematics of archaea and bacteria, 1-3.

Garrity GM, Holt JM, Huber H, Stetter KO, Greene AC, Patel BK, Caccavo F, Allison MJ, MacGregor BJ, Stahl DA. 2001. Phylum BIX. Deferribacteres phy. nov, p 465-471, Bergey’s manual of systematic bacteriology.

Tamazawa S, Mayumi D, Mochimaru H, Sakata S, Maeda H, Wakayama T, Ikarashi M, Kamagata Y, Tamaki H. 2017. Petrothermobacter organivorans gen. nov., sp. nov., a thermophilic, strictly anaerobic bacterium of the phylum Deferribacteres isolated from a deep subsurface oil reservoir. Int J Syst Evol Microbiol 67:3982-3986.

Rees GN, Patel BK, Grassia GS, Sheehy AJ. 1997. Anaerobaculum thermoterrenum gen. nov., sp. nov., a novel, thermophilic bacterium which ferments citrate. Int J Syst Evol Microbiol 47:150-154.

Bhandari V, Gupta RS. 2014. Molecular signatures for the phylum (class) Thermotogae and a proposal for its division into three orders (Thermotogales, Kosmotogales ord. nov. and Petrotogales ord. nov.) containing four families (Thermotogaceae, Fervidobacteriaceae fam. nov., Kosmotogaceae fam. nov. and Petrotogaceae fam. nov.) and a new genus Pseudothermotoga gen. nov. with five new combinations. Antonie van Leeuwenhoek 105:143-168.

Dahle H, Garshol F, Madsen M, Birkeland NK. 2008. Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. Antonie van Leeuwenhoek 93:37-49.

Sherry A, Gray N, Ditchfield A, Aitken C, Jones D, Röling W, Hallmann C, Larter S, Bowler B, Head I. 2013. Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. Int Biodeter Biodegr 81:105-113.

Liang B, Wang L-Y, Mbadinga SM, Liu J-F, Yang S-Z, Gu J-D, Mu B-Z. 2015. Anaerolineaceae and Methanoseta turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. Amb Express 5:37.
Table 1. The ten most abundant OTUs within each oil seep (marked in light grey) together with their relative abundances in descending order. A core community of 10 OTUs was defined as present in ≥ 97-100 % (prevalence) of the droplets of all three sites. As part of the core, OTUs 21, 36, 52, and 38 were added to the table regardless of their respective relative abundance. OTUs which were not detected in the particular seep or with an abundance <0.009 % are marked as below detect (b.d.). Data and errors depict the mean and the standard deviation of the relative abundances of the respective organism in all droplets of one site.
| OTU | Family               | Genus               | Prevalence | La Brea  | McKittrick | Pitch Lake |
|-----|----------------------|---------------------|------------|----------|------------|------------|
| 1   | Hydrogenophilaceae   | Tepidiphilus        |            | 0.01 (± 0.004) | 3.8 (± 0.16) |            |
| 2   | Porphyromonadaceae   | unclassified        | 100 %      | 1.01 (± 0.08) | 0.66 (± 0.04) | 0.9 (± 0.06) |
| 3   | Comamonadaceae       | unclassified        |            | 1.47 (± 0.2)  | 0.66 (± 0.08) |            |
| 4   | Methanotrachaceae    | Methanothrix        | 1.8 (± 0.13) | b.d.     | b.d.       |            |
| 5   | Pseudomonadaceae     | Pseudomonas         | 0.93 (± 0.24) | 0.03 (± 0.01) | b.d.       |            |
| 6   | Clostridiales_Incertae_Sedis XI | Soehngenia | 97 %   | 0.01 (± 0.002) | 0.55 (± 0.05) | b.d.       |
| 7   | Betaproteobacteria (unclassified) | unclassified | 100 % | 0.7 (± 0.05) |            |            |
| 8   | Desulfobulbulaceae   | Desulfoprunum       |            | 0.01 (± 0.002) | 0.03 (± 0.001) |            |
| 9   | Syntrophobacteraceae | unclassified        |            | 0.01 (± 0.002) | 0.05 (± 0.003) | 0.86 (± 0.06) |
| 10  | Woesearchaeta (unclassified) | unclassified |            | 0.51 (± 0.08) | 0.2 (± 0.01) |            |
| 11  | Syntrophorhabdus     | unclassified        | 0.38 (± 0.06) | b.d.     | b.d.       |            |
| 12  | Comamonadaceae       | unclassified        |            | 0.01 (± 0.002) | 0.51 (± 0.11) |            |
| 13  | Hydrogenophilaceae   | Tepidiphilus        | 0.43 (± 0.04) | 0.01 (± 0.002) | b.d.       |            |
| 14  | Hydrogenophilaceae   | Thiobacillus        |            | 0.4 (± 0.03)  | b.d.       |            |
| 15  | Deferrribacteraceae  | unclassified        | 0.02 (± 0.003) | 0.08 (± 0.01) | 0.55 (± 0.11) |            |
| 16  | Synergistaceae       | Anaerobaculum       | 0.32 (± 0.003) | 0.01 (± 0.003) | b.d.       |            |
| 17  | Atribacteria (unclassified) | unclassified | 100 % | 0.16 (± 0.02) | 0.03 (± 0.004) | 0.2 (± 0.01) |
| 18  | Gammaproteobacteria (unclassified) | unclassified |            | 0.45 (± 0.13) | b.d.       | b.d.       |
| 19  | Synergistaceae       | Thermovirga         |            | 0.32 (± 0.005) | 0.01 (± 0.001) | b.d.       |
| 20  | Deferrribacteraceae  | Coldtherovibrio     |            | 0.32 (± 0.005) | 0.01 (± 0.001) | b.d.       |
| 21  | Bacteroidaceae       | Bacteroides         |            | 0.23 (± 0.02)  | b.d.       |            |
| 22  | Bacteoidaceae        | Bacteroides         |            | 0.23 (± 0.02)  | b.d.       |            |
| 23  | Porphyromonadaceae   | unclassified        | 0.01 (± 0.002) | 0.003 (± 0.001) | 0.39 (± 0.02) |            |
| 24  | Synergistaceae       | Syntrophomonile     |            | 0.29 (± 0.04)  | b.d.       |            |
| 25  | Bacillaceae_1        | unclassified        | 0.07 (± 0.001) | 0.19 (± 0.02)  | 0.003 (± 0.0003) |            |
| 26  | Porphyromonadaceae   | Proteiniphilum      |            | 0.26 (± 0.02)  | b.d.       |            |
| 27  | Bacteroidaceae       | Bacteroides         |            | 0.23 (± 0.02)  | b.d.       |            |
| 28  | Bacteroidaceae       | Bacteroides         |            | 0.23 (± 0.02)  | b.d.       |            |
| 29  | Pseudomonadaceae     | Pseudomonas         | 0.93 (± 0.24) | 0.03 (± 0.01)  | b.d.       |            |
| 30  | Clostridiales_Incertae_Sedis XI | Soehngenia | 97 %   | 0.01 (± 0.002) | 0.55 (± 0.05) | b.d.       |
| 31  | Betaproteobacteria (unclassified) | unclassified |            | 0.7 (± 0.05) |            |            |
| 32  | Desulfobulbulaceae   | Desulfoprunum       |            | 0.01 (± 0.002) | 0.03 (± 0.001) |            |
| 33  | Syntrophobacteraceae | unclassified        |            | 0.01 (± 0.002) | 0.05 (± 0.003) | 0.06 (± 0.003) |
| 34  | Atribacteria (unclassified) | unclassified |            | 0.03 (± 0.0044) | 0.07 (± 0.01) | 0.001 (± 0.0003) |
| 35  | Synergistaceae       | Syntrophomonile     |            | 0.13 (± 0.03)  | 0.05 (± 0.01)  | 0.1 (± 0.01) |
| 36  | Bacteroidaceae       | Bacteroides         |            | 0.23 (± 0.02)  | b.d.       |            |
| 37  | Pseudomonadaceae     | Pseudomonas         | 0.93 (± 0.24) | 0.03 (± 0.01)  | b.d.       |            |
| 38  | Bacillaceae_1        | unclassified        | 97 %       | 0.06 (± 0.01)  | 0.02 (± 0.002) | 0.1 (± 0.01) |

Relative abundance of OTUs per oil seep
Figures 629

Fig. 1 CLSM fluorescence images of natural water inclusions (black) dispersed in oil (green) from McKittrick (A, C) and La Brea (B) oil samples. Bright green dots represent microbial cells stained with Syto™9. Images A and B show a two-dimensional view and image C shows a three-dimensional view of different water droplets.
Fig. 2 Box plots of total cell counts of isolated water droplets from the three different oil seeps: Pitch Lake (Trinidad and Tobago), McKittrick (California, USA), and La Brea Tar Pits (California, USA). In total 30 droplets (10 of each oil seep) were counted with a Thoma chamber.
Fig. 3 CLSM fluorescence micrograph of a water droplet isolated from the La Brea Tar Pits. The cells were stained with Syto™9 and propidium iodide. Membrane-intact cells appear green, whereas membrane-damaged cells are stained red.

Fig. 4 Distribution of membrane-intact (black bars) and membrane-damaged cells (hatched bars) in water droplets of the three tested oil seeps. In total, 197 cells were evaluated from Pitch Lake droplets, 1394 from McKittrick droplets, and 1564 from La Brea droplets.
Fig. 5 Measured ATP content in water droplets extracted from the three oil seeps.

Bray-Curtis Dissimilarity
(stress = 0.09874251)

Fig. 6 Non-metric multidimensional scaling (NMDS) plot of beta diversity of all 32 water droplet communities from the three oil seeps. NMDS was calculated based on Bray-Curtis dissimilarity indices with stress level = 0.098. Dashed ellipses display the normal distribution, the solid ellipses display the t-distribution.
Bray-Curtis Dissimilarity
(stress = 0.09874251)

- La Brea
- McKittrick
- Pitch Lake