Natural bitumen is an extraordinary habitat for microorganisms

Dissertation

zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

- Dr. rer. Nat. -

vorgelegt von

Mark Pannekens

geboren in Duisburg

Aquatische Mikrobiologie - Fakultät für Chemie

der Universität Duisburg-Essen

2021

DOI: 10.17185/duepublico/75884
URN: urn:nbn:de:hbz:465-20220503-093853-6

Die vorliegende Arbeit wurde im Zeitraum von Dezember 2015 bis August 2020 im Arbeitskreis von Herrn Prof. Dr. Rainer U. Meckenstock in der Fakultät für Chemie, Umweltmikrobiologie und Biotechnologie, Aquatische Mikrobiologie der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 11.04.2022

Gutachter:	Prof. Dr. Rainer U. Meckenstock
	Prof. Dr. Hans-Curt Flemming
	Prof. Dr. Kirsten Küsel
Vorsitzender:	Prof. Dr. Maik Walpuski

Für meinen Sohn Jonas Jan

“Everybody needs a friend”

- Bob Ross -

Acknowledgement

First, I would like to thank Prof. Dr. Rainer Meckenstock for giving me the opportunity to be part of this fascinating project. Rainer, I´m deeply thankful for all the trust in my work and the freedom to include own ideas to the project. I appreciate your advice and guiding during the years, especially our countless scientific discussions. It was an amazing journey, which has influenced me sustainably.

Also, I would like to thank Prof. Dr. Hans-Curt Flemming for being the co-examiner of my dissertation. This work is strongly influenced by your lectures and talks about microbial biofilms during my Water Science course and even beyond.

Further, I would like to thank the European Research Council, for funding this project and making this work possible.

I´m truly grateful for all the help and assistance by Michelle Kulbatzki, Lucienne Schlinkert, and Arne Meier during my PhD study.

Special thanks to Lisa Voskuhl and Dr. Verena Brauer for their support in bioinformatic questions, the input during our countless meetings, and the fun time besides the lab. It was really a pleasure to work together with you on our project.

I would like to thank Dr. Hubert Müller for his advises and help during several writing processes and for the enjoyable time in our office and besides our work. Furthermore, I would like to thank Dr. Christian Thyssen, Dr. Jan Frösler, and Dr. Marcel Schulte for the trainings in the lab regarding the IC, Delta Ray, CLSM, and Luminometer and for many hours of explaining calculations and even more hours of scientific discussions. I enjoyed the sessions at the squash court or sometimes in a pub very much.

Further, I want to thank Mrs. Astrid Dannehl. Thank you very much, Astrid, for always supporting me during this work. I really appreciate the trainings in microbial methods, and your help at ordering equipment, which saved me many hours during the years. You are indeed a walking lexicon for microbial work.

In addition, I would like to thank Dr. Jost Wingender. Jost, thank you very much for all the advises and answers to questions during the years, which influenced this work a lot.

Also, I want to thank all my colleagues for the interesting, exciting, and fantastic journey of the past years. Especially Dr. Martin Mackowiak for the fun time in our office and playing

foosball, Meike Arnold, Dr. Daniel Köster, Till Bornemann, Dr. Beate Krok, Dr. Philip Weyrauch, Lena Carstens, Dr. Ivana Kraiselburd, Felix Löffler, and Dr. Natascha Teubner for your support in- and besides the lab. Thank you very much, it was a pleasure.

Ganz besonders möchte ich meiner Familie und speziell meiner Mutter für die großartige Unterstützung über all die Jahre hinweg danken. Ohne eure Hilfe hätte ich diesen Weg niemals einschlagen können. Mein größter Dank gilt meiner Frau Helena. Du warst in all den Jahren auch in stressigen Zeiten immer für mich da, dafür bin ich dir unendlich dankbar. Danke für die wundervollen Jahre an deiner Seite, ich blicke voller Vorfreude auf die noch kommenden Jahre zusammen mit dir und unserem Sohn Jonas.

Table of content

Abbreviations

ACS	American Chemical Society
ATP	adenosine triphosphate
CLSM	confocal laser scanning microscopy
Con A	Concanavalin A
CRISPR	clustered regularly interspaced short palindromic repeats
CSL 3	*Oncorhynchus* keta Lectin
CT	computed tomography
DIC	dissolved inorganic carbon
DFG	German Research Foundation
EOR	enhanced oil recovery
EPDM	ethylene propylene diene monomer
EPS	extracellular polymeric substances
ERC	European Research Council
FTICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
GS II	Griffonia Simplicifolia Lectin II
IC	ion chromatography
IUPAC	International Union of Pure and Applied Chemistry
OTU	operational taxonomic unit
OWTZ	oil-water transition zone
PAH	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction
PNA	Peanut agglutinin
RSIL	reverse stable isotope labeling
SBA	Soybean agglutinin
SOP	standard operating procedure
SRB	sulfate-reducing bacteria
TOC	total organic carbon
UEA	Ulex Europaeus Agglutinin I
VPDB	Vienna Pee Dee Belemnite
WGA	Wheat germ agglutinin

Abstract

Microorganisms are found in almost every petroleum reservoir with moderate temperatures. Moreover, most of the world's petroleum deposits are dominated by biologically altered heavy oil and bitumen. Most biodegradation in deep subsurface reservoirs takes place at the oil-water transition zone (OWTZ). This is the contact zone of a petroleum-bearing layer and an underlaying water leg. However, investigations of natural occurring processes in deep petroleum reservoirs are challenging due to a lack of undisturbed non-mixed samples. Furthermore, artificial cultivation causes changes in the microbial community composition and metabolic rates. Fortunately, a recent study found microbial life enclosed in microliter-sized water droplets dispersed in bitumen of the Pitch Lake, located in Trinidad and Tobago. These findings suggest that microbial life, and thus biodegradation additionally takes place in water enclosures independently of the OWTZ. These micro habitats are an exceptional opportunity to get representative insights in microbial life in deep subsurface petroleum reservoirs. However, the question occurs whether microbially inhabited water droplets are a generic trait in bitumen reservoirs rather than a unique phenomenon of the Pitch Lake.

This study investigates microbial life enclosed in microliter-sized water droplets in bitumen, and its impact on the overall biodegradation process. Hence, samples were taken from three different bitumen seeps: The Pitch Lake (La Brea, Trinidad and Tobago), the La Brea Tar Pits (Los Angeles, California, USA), and an unnamed oil seep on the McKittrick oil field (McKittrick, California, USA). First investigations regarding dispersed water revealed the presence of 1-10 µl sized water droplets in all three tested bitumen seeps. Additional three-dimensional computed tomography scans of Pitch Lake bitumen revealed a multitude of smaller water inclusions <0.1 µl and only a minor fraction of 0.2 % of larger droplets. Thereby, these enclosed water droplets generate a bitumen-water interface of 1134 cm^2 per liter bitumen on average, strongly enlarging the overall bioavailable bitumen-water interface in these reservoirs. Examinations of extracted water droplets from the three seeps showed astonishing cell densities of up to 10^9 cells ml^{-1} and an average cell number of $2.0 \times 10^8 \pm 3.6 \times 10^7$ cells ml^{-1} in all analyzed droplets. Viability tests with LIVE/DEAD staining proved that 53 % of the present microorganisms counted as membrane intact. Additionally, ATP determinations showed concentrations comparable to those of other subsurface ecosystems, confirming the presence of metabolic activity. Microbial community analyses based on 16S rRNA gene amplicon sequencing of water droplets revealed typical anaerobic microorganisms known to be involved in the oil-degradation process of other petroleum reservoirs around the world. Although the sampling sites were located at far apart

places, all three bitumen seeps shared several operational taxonomic units (OTUs). Besides that, a core community of 8 OTUs was present in 97 to 100 % of all analyzed droplets. *In situ* cell staining in water droplets followed by microscopic investigations, as well as lectin staining of the bitumen surface in artificial microcosm setups, showed microbial cells attached to the bitumen surface imbedded by fluorescent structures. Furthermore, 16S rRNA gene amplicon sequencing of the bitumen surface revealed differences in the microbial community compositions compared to samples from the aqueous phase. The results all together strongly support the common idea of biofilm formation as the prevailing form of life within petroleum reservoirs. Degradation rates of Pitch Lake bitumen were measured by applying "reverse stable isotope labeling". This method enables precise measurements of relatively low mineralization rates in the ng range. For this purpose, freshly taken bitumen samples were overlain with artificial brackish water and incubated for 945 days. The obtained data revealed a mineralization rate of 9.4−38.6 mmol CO_2 per liter bitumen and year.

This study confirms that inhabited, microliter-sized water droplets are a common feature in bitumen reservoirs. It was shown that the densely populated micro habitats harbor active and diverse microbial communities which are presumably organized in biofilms. In addition, evidence was provided that these water droplets considerably enlarge the bitumen-water interface and could play an important role in the overall degradation process in deep subsurface petroleum reservoirs regarding geological times scales. This study provides new insights into life in natural bitumen reservoirs away from the OWTZ and builds the foundation for further research on microbial ecology, syntrophic interactions, and metabolic processes in deep subsurface petroleum reservoirs.

Zusammenfassung

Mikroorganismen können in fast allen Erdöllagerstätten mit gemäßigten Temperaturen nachgewiesen werden. Weltweit werden die meisten Erdöllagerstätten von biologisch abgebautem Schweröl und Bitumen dominiert. Der größte Teil des biologischen Abbaus in tiefen unterirdischen Lagerstätten findet an der Öl-Wasser-Übergangszone (eng. OWTZ) statt. Dies ist eine Kontaktzone zwischen einer erdölführenden Schicht und einer darunter liegenden Wasserschicht. Die Untersuchung natürlicher Prozesse in tiefen Erdöllagerstätten ist aufgrund mangelnder ungestörter und nicht durchmischter Proben schwierig. Zusätzlich führt eine künstliche Kultivierung zu Veränderungen in der Zusammensetzung von natürlich vorkommenden mikrobiellen Gemeinschaften und Abweichungen in den Stoffwechselraten. In einer aktuellen Studie wurden Mikroorganismen in mikrolitergroßen Wassertropfen gefunden, die im Bitumen des Pitch Lake in Trinidad und Tobago verteilt waren. Diese Einschlüsse deuten darauf hin, dass mikrobielles Leben und somit biologischer Abbau auch unabhängig von der OWTZ in Wassereinschlüssen vorkommen. Dadurch sind diese eingeschlossenen und separierten Mikrohabitate eine außergewöhnliche Möglichkeit allgemeine Einblicke in das mikrobielle Leben in tiefen unterirdischen Erdöllagerstätten zu erhalten. Es bleibt jedoch zu klären, ob mikrobiell besiedelte Wassertropfen ein generelles Merkmal in Bitumenlagerstätten sind oder nur eine Ausnahme im Pitch Lake.

Diese Studie untersucht mikrobielles Leben in mikrolitergroßen Wassertropfen in Bitumen und seine Auswirkungen auf den gesamten biologischen Abbauprozess. Dafür wurden Proben aus drei unterschiedlichen Bitumenlagerstätten entnommen: Dem Pitch Lake (La Brea, Trinidad und Tobago), den La Brea Tar Pits (Los Angeles, Kalifornien, USA) und einer unbenannten Quelle auf dem McKittrick-Ölfeld (McKittrick, Kalifornien, USA). Erste Untersuchungen bezüglich kleinster Wassereinschlüsse bestätigten das Vorkommen von 1-10 µL großen Wassertropfen in allen drei untersuchten Bitumenlagerstätten. Zusätzliche Untersuchungen mittels dreidimensionaler Computertomographie von Bitumen aus dem Pitch Lake zeigten eine große Anzahl kleinerer Wassereinschlüsse <0,1 µL und lediglich einen Anteil von 0,2 % mit größerem Volumen. Die eingeschlossenen Wassertropfen erzeugen eine Bitumen-Wasser-Grenzfläche von durchschnittlich 1134 cm^2 pro Liter Bitumen, was die gesamte bioverfügbare Grenzfläche in Lagerstätten deutlich vergrößert. Weitere Untersuchungen von extrahierten Wassertropfen aus den drei Lagerstätten ergaben erstaunliche Zelldichten von bis zu 10^9 Zellen mL^{-1} und eine durchschnittliche Zellzahl von $2{,}0 \times 10^8 \pm 3{,}6 \times 10^7$ Zellen mL^{-1}. Die Lebensfähigkeit der vorhandenen Zellen wurde mittels LIVE/DEAD-Färbung überprüft. Die Auswertung der Ergebnisse ergab, dass 53 % der

vorhandenen Mikroorganismen als membran-intakt und somit als lebend galten. Darüber hinaus ergaben ATP-Messungen Konzentrationen, die vergleichbar sind mit denen anderer unterirdischer Ökosystemen, was das Vorhandensein metabolisch aktiver Mikroorganismen bestätigt. Die Analyse der mikrobiellen Gemeinschaft einzelner Wassertropfen auf der Grundlage von 16S rRNA-Gen-Amplikonsequenzierung zeigte typische anaerobe Mikroorganismen, deren Beteiligung an Abbauprozessen in Erdöllagerstätten auf der ganzen Welt bereits bekannt ist. Obwohl die Probenahmestellen an voneinander weit entfernten Orten lagen, teilten alle drei Bitumenlagerstätten gleich mehrere operative taxonomische Einheiten (eng. OTUs). Eine Gruppe von 8 OTUs war in 97 bis 100 % aller untersuchten Tropfen vorhanden. *In-situ*-Zellfärbung in Wassertropfen, gefolgt von mikroskopischen Untersuchungen, sowie die Färbung der Bitumenoberfläche in angelegten Mikrokosmen mit fluoreszenzmarkierten Lektinen, zeigten mikrobielle Zellen auf der Bitumenoberfläche, die nach der Lektinfärbung zusätzlich von fluoreszierenden Strukturen umgeben waren. Die 16S rRNA-Gen-Amplikonsequenzierung der Bitumenoberflächen zeigte deutliche Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaft im Vergleich zu Proben aus der wässrigen Phase. Die Ergebnisse untermauern die gängige Vorstellung der Biofilmbildung als vorherrschende Form des Lebens in Erdöllagerstätten. Darüber hinaus wurden die Abbauraten von Pitch Lake Bitumen durch "reverse stable isotope labeling" gemessen. Diese Methode ermöglicht die präzise Messung von relativ geringen Mineralisierungsraten im ng-Bereich. Dazu wurden frisch entnommene Bitumenproben mit künstlichem Brackwasser überlagert und 945 Tage lang inkubiert. Die Auswertung der erhaltenen Daten ergaben eine Mineralisierungsrate von 9,4-38,6 mmol CO_2 pro Liter Bitumen und Jahr.

Diese Studie bestätigt, dass bewohnte, mikrolitergroße Wassertropfen ein gemeinsames Merkmal von Bitumenlagerstätten sind. Es konnte gezeigt werden, dass diese dicht besiedelten Mikrohabitate aktive und vielfältige mikrobielle Gemeinschaften beherbergen, die vermutlich in Biofilmen organisiert sind. Darüber hinaus wurde nachgewiesen, dass die Wassertropfen die Bitumen-Wasser-Grenzfläche erheblich vergrößern und aufgrund der festgestellten Mineralisierungsrate, über geologische Zeiträume hinweg, eine wichtige Rolle im gesamten Abbauprozess in tiefen unterirdischen Erdöllagerstätten spielen könnten. Diese Studie bietet neue Einblicke in das mikrobielle Leben in natürlichen Bitumenlagerstätten abseits der OWTZ und bildet die Grundlage für weitere Forschung zur mikrobiellen Ökologie, zu syntrophen Interaktionen und Stoffwechselprozessen in tiefen unterirdischen Erdöllagerstätten.

1 General introduction

1.1 Natural petroleum reservoirs

Petroleum is one of the most important resources of our daily life.[1, 2] Thereby, the usage of fossil fuel as an energy source led to increased carbon dioxide emissions which negatively contributes to global warming.[3] Natural petroleum originates from biological residues which were formed by high pressure and heat in the deep subsurface over geological time scales. Crude oil (a none degraded form of petroleum) consists mainly of four main fractions: saturated and aromatic hydrocarbons, resins and asphaltenes. These factions in turn are a complex mixture of thousands of different compounds, mostly consisting of aliphatic compounds of the paraffin series, aromatic compounds of benzene series, and naphthenic compounds of the polymethylene or cycle-paraffin series. In addition, fatty acids, phenols, resinous compounds, asphaltenes, naphthenic acids, sulfones, thiophenes, sulfonic acids, sulfoxides, pyridines, and others are present in crude oils.[4, 5] Crude oil is mostly pumped from deep subsurface oil reservoirs as an emulsion of crude oil and water. This production water can either be formation water, originating from an underlaying water layer below the oil reservoir, or anthropogenic injected seawater or fresh water for enhanced oil recovery.[6, 7]

1.2 Microbial degradation of petroleum

Besides chemical processes, most petroleum reservoirs are exposed to biological degradation. Thereby, more than half of the world's largest petroleum accumulations are biodegraded and dominated by biologically altered heavy oil or bitumen.[8-10]

In 1926, amongst others, sulfate reducing bacteria (SRB) were found to be responsible for the hydrogen sulfide production in formation water of an oil reservoirs in the southeastern and southwestern of Illinois, USA.[11] In 1945, evidence in petroleum hydrocarbon oxidation by microorganisms was provided by showing that, microorganisms were able to transform and oxidize certain compounds present in oil reservoirs.[5] Since then, countless studies have found microorganisms to be present in oil and water samples from different crude oil reservoirs around the world.[7, 12-14] Further, it is accepted, that more than one microbial consortium is responsible for the degradation of different petroleum components.[15] The presence of bacteria in petroleum samples from the deep subsurface indicates that many species have evolved and adapted in this environment. Nevertheless, some species may have originated from petroleum free soils and survived the seeping of oil into their habitat or were introduced anthropogenically during oil production.[12, 16-18] Generally, petroleum reservoirs have a low

redox potential and therefore harbor mostly anaerobic and facultatively anaerobic microorganisms.[3] The microbial degradation of crude oil in deep subsurface oil reservoirs negatively affects the worldwide oil resources by lowering the oil quantity and quality. Thereby, biodegradation causes changes in physical properties like an increase in density, viscosity, acidity, sulfur-, asphaltene-, and metal content. Microbial degradation of hydrocarbons leads to a production of acyclic and cyclic hydrocarbons, saturated and aromatic carboxylic acids, and phenols and changes the oil to gas ratios. These changes increase the recovery and refining costs.[8-10, 13, 19] In addition, the oxidation of oil constituents like saturated hydrocarbons (C_{6+}) during biodegradation causes an increase of the polar fraction such as resins and asphaltenes which decrease the American Petroleum Institute (API) gravity. API gravity quantifies the actual degradation status, which correlates with the economic value of the oil. Nondegraded light marine oils generally have an API gravity of 36-38, heavily degraded oils however have an API gravity of 20, and super-heavy oils an API gravity value of 10 or less.[8, 13, 19] Many biological and chemical parameters affect the degree of biodegradation: The ratio of water leg to oil leg, the oil-water transition zone (OWTZ), water salinity, the overall oil volume, the prevailing temperature, and oil compound composition influence the overall degradation process.[7, 20] Deep subsurface petroleum reservoirs are usually not connected to water of meteoric cycles. Therefore, nitrate and oxygen are only available in low amounts.[9] Most biodegradation in deep subsurface reservoirs occurs under anaerobic conditions.[21] Biodegradation generally occurs in natural oil reservoirs up to 82 °C.[14, 22] Further, oil reservoirs at lower temperatures are more affected by biodegradation.[19] Petroleum components are degraded in the following order: n-alkanes, alkylcyclohexanes, acyclic isoprenoid alkanes, bicyclic alkanes–steranes–hopanes. The most persistent group of compounds are aromatic steroid hydrocarbons and polycyclic aromatic hydrocarbons (PAH), which are only removed in heavily biodegraded oil.[6, 9, 13] Biodegradation of crude oil components was proven by the detection of metabolites characteristic for anaerobic hydrocarbon degradation.[21] The relative abundance of specific biomarkers like n-alkanes, norhopanes, methyl or 25-norhopane, are characteristic compounds to evaluate the extent of biodegradation in oil reservoirs. Thereby, dimethylnaphthalenes or 25-norhopanes are only present in super-heavy oils.[13, 23] The current paradigm assumes that the present microbial communities originate from underlaying water layers were the major biodegradation takes place at the OWTZ.[6-8, 24, 25]

1.3 Natural bitumen

Successive crude oil biodegradation leads to bitumen. Bitumen consists of high molecular weight hydrocarbons of a complex and undefined chemical composition.[20] In terms of elemental composition, bitumen consists mainly of carbon (~80-88 %), hydrogen (~8-11 %), oxygen (~1-15 %), sulfur (1-8 %), and nitrogen (~1 %). In comparison to crude oil, bitumen mostly consists of cyclic and aromatic hydrocarbons, resins or asphaltenes. Asphaltene constituents are the highest molecular weight and most polar part in crude oil and are ranging from a few hundred to several million.[26] They are defined as the oil fraction that dissolve in toluene and precipitates in n-alkane solvents. Compared to the entire petroleum or bitumen, asphaltenes have a lower hydrogen content of around 4.8-8 % and a higher content of nitrogen, sulfur, oxygen, vanadium, and nickel. Asphaltenes do not contain many straight chains but are generally rich in ring groups ranging from 1-7 rings in a single cluster.[27] These complex mixtures of different compounds can last for long time periods.[28] However, resins include a variety of different hydrocarbon types and functional groups including thiophene, benzothiophene, and dibenzothiophene systems, hydrogenbonded hydroxyl groups, pyrrole N-H functions, ester functions, acid functions, carbonyl functions, and sulfur-oxygen functions. Resins consists of carbon (85 ± 3 %), hydrogen (10.5 ± 1 %), nitrogen (0.5 ± 0.15 %), oxygen (1.0 ± 0.2 %) and sulfur (ranging from 0.4 to 5.1 %).[26] The complex bitumen matrix makes it impossible to mimic natural bitumen artificially for experimental setups with isotopically labeled compounds. One major use of bitumen is as adhesive in road construction, but also as protection material for roofs and pipes, and in the automobile industry. Therefore, the mined bitumen gets refined and mixed with the appropriate sands and gravels.[28]

The two largest single bitumen deposits are the foreland basins east of Venezuela (1,200 billion barrels) and the tar sand deposits (900 billion barrels) in Alberta at the western part of Canada.[10] The largest natural bitumen surface reservoir, the so-called Pitch Lake, is in Trinidad and Tobago. The Pitch Lake is a natural bitumen lake located in the southwestern of the island Trinidad and covers about 40 ha with a depth of around 80 m and is fed by an underlying oil reservoir in approx. 1500 m depth (Figure 1A).[29] Pitch Lake bitumen mostly consists of hydrocarbons (23.3 %, wt.), benzene resins (18.6 %, wt.), alcohol–benzene resins (15.9 %, wt.), resins (34.5 %, wt.), asphaltenes (42.2 %, wt.), and sulfur (around 6 %, wt.).[30] In the deeper subsurface reservoir, the present pitch is mixed at high pressure with mud and gases. Thereby, the lighter portion evaporates, leaving a liquid asphalt residue, which is characterized by low water activity, recalcitrant carbon substrates, and toxic chemical

compounds.[31] Another natural surface asphalt reservoir are the Rancho La Brea tar pits (Los Angeles, CA, USA). These are commonly called tar pits, but the present petroleum hydrocarbons are correctly referred to as natural asphalt or bitumen (Figure 1B). The seeps are fed by natural bitumen originating from underlying oil sands. The trapped bitumen is moved by methane under pressure through cracks to the surface in the Pleistocene alluvium, thereby natural seeps with several square meters in area and around 9 to 11 m in depth were formed.[16, 32] For many years, bitumen seeps were believed to be biostatic due to toxic water-soluble hydrocarbons such as naphthenic acids, the high heavy metal content, high temperatures, or the low water activity.[31, 33]

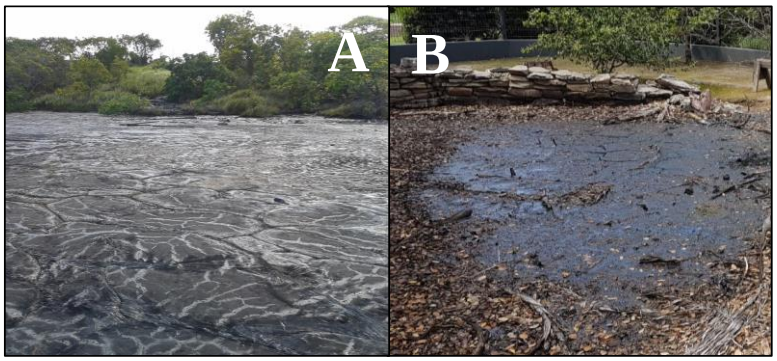


Figure 1 Two natural asphalt seeps. A: Pitch Lake located in the southwest of Trinidad (Trinidad and Tobago). B: The Rancho La Brea Tar Pits located in Los Angeles (USA).

1.4 Microbial life in water enclosures in bitumen

Nevertheless, many different active or culturable microorganisms were found to be present in liquid bitumen samples.[28, 31] In 2007, Kim and Crowley (2007) were able to extract DNA for 16S rRNA gene sequencing from bitumen of the Rancho La Brea Tar Pits in Los Angeles (CA, USA), the analysis revealing the presence of archaea and bacteria. They proposed, that small water pockets could be the main site for microbial growth within the bitumen pits.[16] In 2014, Meckenstock *et al.,* (2014) found tiny water droplets in the size of 1-3 microliter enclosed in the bitumen body far away from the oil-water transition zone. Isotope analysis revealed an origin from the deep subsurface. These water enclosures or droplets contained microorganisms and were believed to function as tiny microhabitats midst the oil phase. Further analysis revealed complex microbial communities enclosed in these tiny droplets. Further, analysis of extracted water revealed functionalized low–molecular weight compounds, which provides an indirect proof for biodegradation. These findings strongly indicate active microbial biodegradation within the bitumen phase.[34]

Nonetheless, little is known about these enclosed water droplets, the present microorganisms, and their role in natural bitumen reservoirs, leading to further questions. Are the present microorganisms alive and active or only steady state residues from an underlaying

water body? How many microorganisms are living in these tiny habitats, and what is their natural extent on the overall biodegradation process in natural bitumen reservoirs? How common are these enclosed inhabited water droplets in natural bitumen reservoirs, are they an extraordinary and exotic phenomenon only occurring in Pitch Lake bitumen or rather a norm in bitumen reservoirs?

1.5 Microbial processes in petroleum reservoirs

Several microbial studies found a huge range of different bacterial and archaeal taxa and a variety of different metabolisms present in deep subsurface oil reservoirs.[3, 12, 35] The most common metabolisms found in oil reservoirs are fermentation, sulfate reduction, acetogenesis, and methanogenesis. Further, nitrate-, manganese-, and iron-reducing microorganisms are present in many oil reservoirs, but it is unclear how prevalent these metabolisms are, since they are usually limited by the availability of the corresponding electron acceptors.[3, 7, 36, 37] Therefore, fermentation, sulfate reduction, and methanogenesis are believed to be the main processes involved in the overall hydrocarbon oxidation in petroleum reservoirs.[38]

1.6 Syntrophic interactions in petroleum reservoirs

Fermenting microorganisms are frequently detected in many oil fields and are often involved in hydrocarbon degradation processes. Those microorganisms can utilize organic compounds both, as electron donor as well as electron acceptor. Thereby, fermenters can degrade larger organic compounds like carbohydrates from cellular debris, organic acids, or amino acids into smaller molecules.[13, 37, 39] Common fermentation products are butyrate, propionate, acetate, formate, lactate, hydrogen, and carbon dioxide, which are in turn used as a nutrient source form other groups of microorganisms e.g., SRB or methanogenic archaea.[9, 12, 13, 40-44] Members of the candidate phylum of Atribacteria often are part of the microbial community in petroleum reservoirs, some of them may have the ability to ferment short-chain n-alkanes into fatty acids, which could be consumed by secondary degraders or used for biosynthesis. Therefore, Atribateria as possible fermenting microorganisms may play an important role in the degradation of oil reservoirs.[45]

But not only fermenters are able to break down organic compounds. SRB have a wide spectrum of substrates and are known for complete alkane (C_3-C_{20}) oxidation to carbon dioxide and to degrade short and long chain fatty acids.[38, 43, 46] Further, sulfate reducers are known for aromatic hydrocarbon degradation such as benzene, toluene, naphthalene, 2-methylnaphthalene, phenanthrene, ethylbenzene, and xylene (table 1).[4, 43, 47-50]

Table 1 Biodegradation of hydrocarbons in petroleum reservoirs by SRB

Substrate	Stoichiometric equations	
Benzene	$C_6H_6 + 3H_2O + 3.75SO_4^{2-} \rightarrow 6HCO_3^- + 3.75HS^- + 2.25H^+$	[48]
Toluene	$C_7H_8 + 4.5SO_4^{2-} + 3H_2O \rightarrow 7HCO_3^- + 2.5H^+ + 4.5HS^-$	[43]
Decane	$C_{10}H_{22} + 7.75SO_4^{2-} + 5.5H^+ \rightarrow 10HCO_3^- + 7.75H_2S + H_2O$	[51]
Naphthalene	$C_{10}H_8 + 6SO_4^{2-} + 6H_2O \rightarrow 10HCO_3^- + 6HS^- + 4H^+$	[48]
2-methylnaphthalene	$C_{11}H_{10} + 6.75SO_4^{2-} + 6H_2O \rightarrow 11HCO_3^- + 6.75HS^- + 4.25H^+$	[48]
Phenanthrene	$C_{14}H_{10} + 8.25SO_4^{2-} + 9H_2O \rightarrow 14HCO_3^- + 8.25HS^- + 5.75H^+$	[48, 50]
Hexadecane	$C_{16}H_{34} + 12.25SO_4^{2-} + 8.5H^+ \rightarrow 16HCO_3^- + 12.25H_2S + H_2O$	[4, 49]

Generally, SRB can be grouped into incomplete and complete degraders, which oxidize organic compounds to acetate or completely to carbon dioxide, respectively. In addition, many SRB could change to fermentation if sulfate gets depleted.[52] However, if sulfate is available, SRB compete with proton-reducing acetogenic bacteria, homoacetogens, and methanogens in anoxic environments with a low redox potential for hydrogen and acetate. Thereby, due to a higher affinity and lower threshold values for hydrogen, SRB rapidly out-competed hydrogen-utilizing methanogens and homoacetogens.[7, 40, 41] In the absence of sulphate, acetate, whether produced by fermentation or indirectly by acetogenesis is consumed by methanogens (Figure 2). At least three groups of microorganisms are involved in this process. Larger or aromatic organic compounds like toluene are degraded by syntrophic acetogenic microorganisms, resulting in the metabolites carbon dioxide, acetate, and hydrogen (equation 1). Produced acetate is converted by acetoclastic methanogenesis into methane and carbon dioxide (equation 2). Hydrogen and carbon dioxide in turn are used by hydrogenotrophic methanogenesis (equation 3).[9, 13, 41-44]

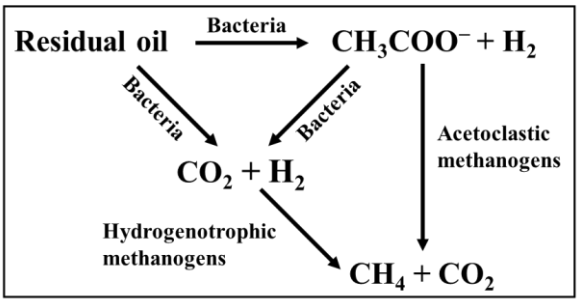


Figure 2 Schematic drawing of the pathways of syntrophic acetogenic bacteria, hydrogenotrophic- and acetoclastic methanogenic archaea involved in degradation of petroleum hydrocarbons (modified from Mbadinga et al., 2011, Head et al., 2010, and Xu et al., 2019).[4, 13, 43]

$$C_7H_8 + 9H_2O \rightarrow HCO_3^- + 3CH_3COO^- + 4H^+ + 6H_2 \quad (1)$$

$$3CH_3COO^- + 3H_2O \rightarrow 3CH_4 + 3HCO_3^- \quad (2)$$

$$6H_2 + 1.5HCO_3^- + 1.5H^+ \rightarrow 1.5CH_4 + 4.5H_2O \quad (3)$$

Even if nitrate-, manganese-, and iron reducers are part of microbial communities in oil reservoirs, their impact on the overall degradation process is still unclear.[3, 7, 36, 37] Although, studies show that they can mineralize benzene or even naphthalene to carbon dioxide.[48] Nevertheless, nitrate, manganese, and ferric iron are mostly not available or only present in very low concentrations, since they are reduced near the surface sediment before they reach the petroleum reservoir or oxidized iron often bounds in unreactive iron silicates.[13, 36]

Even if many microorganisms were found frequently and several metabolic pathways could be linked to certain microorganisms, due to a lack of undisturbed samples (mixed oil-water emulsions through pumping), little is known about the *in situ* syntrophic interactions and complex processes occurring down in deep subsurface reservoirs. Due to the common sampling issues, our knowledge about phylogenetic diversity, metabolic capabilities, ecological roles, community dynamics, and community composition in deep subsurface oil reservoirs remains vague.[3, 8, 21]

Since the tiny water droplets found in the Pitch Lake can be sampled directly from the surface of these seeps without any mixing or other sampling issues, they offer a great chance to get insights in undisturbed natural microbial communities living in bitumen reservoirs which could possibly help to understand the actual processes that are taking place in deep subsurface oil reservoirs.

1.7 Accessing natural biodegradation rates in petroleum reservoirs

Due to the harsh environmental conditions like high pressure and temperatures, salt stress, low water activity, toxic compounds in the oil and water phase, the lack of electron donors like ferric iron, nitrate, or nitrite, in adequate amounts, and the aromatic character of many compounds, biological degradation of crude oil in deep subsurface oil reservoirs is believed to happen in geological time scales.[12, 31, 33] Nonetheless, a multitude of different microorganisms can be found in almost every sample that originate from temperate petroleum reservoirs.[7, 12-14] However, it is still very challenging to obtain reliable data about microbial processes taking place in deep subsurface petroleum reservoirs, since present metabolic rates are rather low and differ strongly when measured in cultures (by factor ~6).[53] A frequently used method to measure the biodegradation of a known substrate is isotope labeling. Thereby, either a substrate or the surrounding water is labeled with ^{2}H, ^{18}O, ^{13}C, or ^{14}C, respectively. Afterwards, the incorporation of heavy isotopes in intermediates, biomass, or metabolites such as $^{13}CO_2$ or $^{14}CO_2$ are measured.[48, 54, 55] Nevertheless, the degradation of crude oil or bitumen by microorganisms leads to a broad range of different metabolic intermediates,

which makes it nearly impossible to precisely target all of these compounds with regards to the surrounding complex petroleum matrix. Another frequently used method is to isotopically label a known substrate and detect the labeled mineralization products. Unfortunately, the complex bitumen matrix contains thousands of different compounds which makes it impossible to artificially create a isotopic labeled crude oil or bitumen matrix.[20, 26-28] Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has a high resolution and can be used to detect the depletion of certain compound classes within the matrix.[34] Nevertheless, a precise detection is very challenging due to the very small mineralization rates during anaerobic bitumen degradation. Therefore, another approach is the direct detection of petroleum mineralization products like carbon dioxide and methane. These can be measured in addition to the total and dissolved organic carbon in the aqueous phase by a multitude of different instruments for instance gas chromatography or TOC-analyzer even in small amounts together with the total and dissolved organic carbon.[56] Nevertheless, depending on the organic load and the metabolic rate, these technics are less precise and have a higher detection limit than isotope labeling approaches.[57]

1.8 Reverse stable isotope labeling

A recently developed substrate independent, sensitive, and noninvasive method could help detecting even lowest mineralization rates of bitumen. The method is called "reverse stable isotope labeling" (RSIL) and is based on the constant dilution of a highly ^{13}C-CO_2 enriched bicarbonate buffer background by the production of CO_2 from hydrocarbon degradation at natural abundance. This method was first implemented by Dong *et al.,* (2017) and since has been used by several follow-up studies to measure turnover rates in the nmol scale.[57-59] Thereby the $^{13}CO_2/^{12}CO_2$-ratio is measured by a Delta Ray Mid infrared spectrometer at a wavelength of 4.3 µm. The obtained stable isotope ratios are expressed in δ notation as $\delta^{13}C$ values in per mil. The device can detect and precisely measure minor changes in the isotopic composition of $\delta^{13}C < 6$ ‰.[59] To avoid isotopic ratio drifts caused by different CO_2 concentrations, the device measures the current CO_2 concentration in samples and dilutes them to a concentration of 380 ppm. The linear correspondence is stable over a broad range from $x(^{13}C) = 6$ % to $x(^{13}C) = 12$ %.[59] For precise calculations, the obtained carbon isotopic ratios ($\delta^{13}C$ values) must be converted into isotope-amount fraction ($x(^{13}C)$) according to Coplen et al., 2011, which proposed to use the delta scale as standard procedure.[60, 61]

A delta value ($\delta(^{h}E)$) is defined as the relative difference between the isotope ratio of a sample and the isotope ratio of a defined international reference (equation 5). An isotope ratio for two isotopes of an element is given by equation 4.

$$R(^{h}E) = \frac{(^{h}E)}{(^{l}E)} \quad (4)$$

$R(^{h}E)$ = isotope ratio
h = heavier isotope, here ^{13}C
l = lighter isotope, here ^{12}C

For carbon, Vienna Pee Dee Belemnite (VPDB) is the anchoring point for defining the delta scale and used as a new international standard reference material with an accepted $^{13}C/^{12}C$ ratio of 0.0111802. It is based on the former Pee Dee Belemnite (PDB), which is no longer available.[62] This standard originates from a marine carbonate of a belemnite fossil (*Belemnitella americana)* from the cretaceous period and was later artificial recreated in Vienna. The natural abundances of heavy and light isotopes of an element can differ by orders of magnitude, leading to very small isotope ratios. Further, isotope fractionation processes, with the exception of hydrogen, are generally small, therefore they are expressed in per mil or ‰ (equation 5).[61]

$$\delta(^{h}E)_{sample,reference} = \left(\frac{R(^{h}E/\ ^{l}E)_{sample} - R(^{h}E/\ ^{l}E)_{reference}}{R(^{h}E/\ ^{l}E)_{reference}}\right) \times 1000 = \left(\frac{R(^{h}E/\ ^{l}E)_{sample}}{R(^{h}E/\ ^{l}E)_{reference}} - 1\right) \times 1000 \quad (5)$$

$\delta(^{h}E)$ = delta value in per mil or ‰
$R(^{h}E)$ = isotope ratio
h = heavier isotope, here ^{13}C
l = lighter isotope, here ^{12}C

A positive value implies that the ratio of heavy to light isotopes is higher in the sample than it is in the standard. The measured δ values of a sample are related to and can be converted into an isotope amount fraction ($x(^{h}E)$) (equation 6).

$$x(^{h}E) = \frac{1}{1 + \frac{1}{\left(\left(\frac{\delta(^{h}E)_{sample}}{1000} + 1\right) \times R(^{h}E/\ ^{l}E)_{reference}\right)}} \quad (6)$$

The isotope amount fraction ($x(^{h}E)$), also called fractional abundance (^{h}F), is defined as the amount or number of one isotope of a chemical element divided by the total amount or number of isotopes of this element (here simplified for two isotopes) (equation 7).

$$x(^{h}E) = \frac{n(^{h}E)}{n(^{l}E) + n(^{h}E)} \quad (7)$$

x(hE) = isotope amount fraction
h = heavier isotope, here ^{13}C
l = lighter isotope, here ^{12}C
n = amount of an element, here C

The isotope amount fraction multiplied by 100 leads to atom percent (atom-%), which is not a conforming unit by the International Union of Pure and Applied Chemistry (IUPAC) (equation 8).

$$\text{atom-\%} = x(^{h}E) \times 100 = \frac{n(^{h}E)}{n(^{l}E) + n(^{h}E)} \times 100 \quad (8)$$

atom-% = atom percent
$x(^{h}E)$ = isotope amount fraction
h = heavier isotope, here ^{13}C
l = lighter isotope, here ^{12}C
n = amount of an element, here C

In terms of a closed system the following mass balance can be considered (equation 9).

$$n_{tot} \times x(^{13}C)_{tot} = n_1 \times x(^{13}C)_1 + n_2 \times x(^{13}C)_2 \quad (9)$$

n_1 = amount of substance compound 1, here bicarbonate buffer background
n_2 = amount of substance compound 2, here actual DIC
n_{tot} = total amount of substance, sum of n_1 and n_2
$x(^{13}C)_1$ = isotope amount fraction compound 1, here bicarbonate buffer background
$x(^{13}C)_2$ = isotope amount fraction compound 2, here bitumen
$x(^{13}C)_{tot}$ = total isotope amount fraction resulting from the relative share of $x(^{13}C)_1$ and $x(^{13}C)_2$

Substituting n_1 and n_2 for n_{tot} and rearranging the mass balance for n_2, enables to calculate the associated amount of dissolved inorganic carbon (DIC) in the sample (equation 10).

$$n_2 = \frac{n_1\left(x(^{13}C)_{tot} - x(^{13}C)_1\right)}{x(^{13}C)_2 - x(^{13}C)_{tot}} \quad (10)$$

By subtracting the initial amount of DIC (start of the experiment) from the actual measured DIC value, the produced amount of CO_2 can be obtained.

Since this method needs no sample preparation, is noninvasive, and inexpensive, it is a promising candidate for detecting and monitoring smallest biological mineralization rates (release of CO_2) of unknown substrates in a complex matrix like bitumen. Furthermore, this method could help analyzing the role of tiny water enclosures enclosed in the bitumen body away from the oil-water transition zone and their impact on the overall biodegradation process.

1.9 Microbial biofilm formation

Microbial biofilms are widespread in nature and represent one of the most successful strategies of life. Hence, most habitats on our planet, except the oceans, are dominated by biofilms.[63-65] Microorganisms use biofilm formation as an adaption to adverse environmental conditions.[63] The official IUPAC definition of biofilms is "Biofilms are aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface". [66] However, due to the broad range of microbial aggregates, flocks, films and clusters, the definition of a biofilm is still controversial.[65, 67, 68] Thereby, EPS form a three-dimensional polymer network which mainly consists of polysaccharides, proteins, nucleic acids, and lipids (Figure 3). Thus, microorganisms only account for around 10 % of the dry mass in most biofilms.[64, 69] The cell density in these complex systems varies across a broad range and can reach population densities of up to 4.9 x 10^{10} cells g^{-1} wet weight.[70]

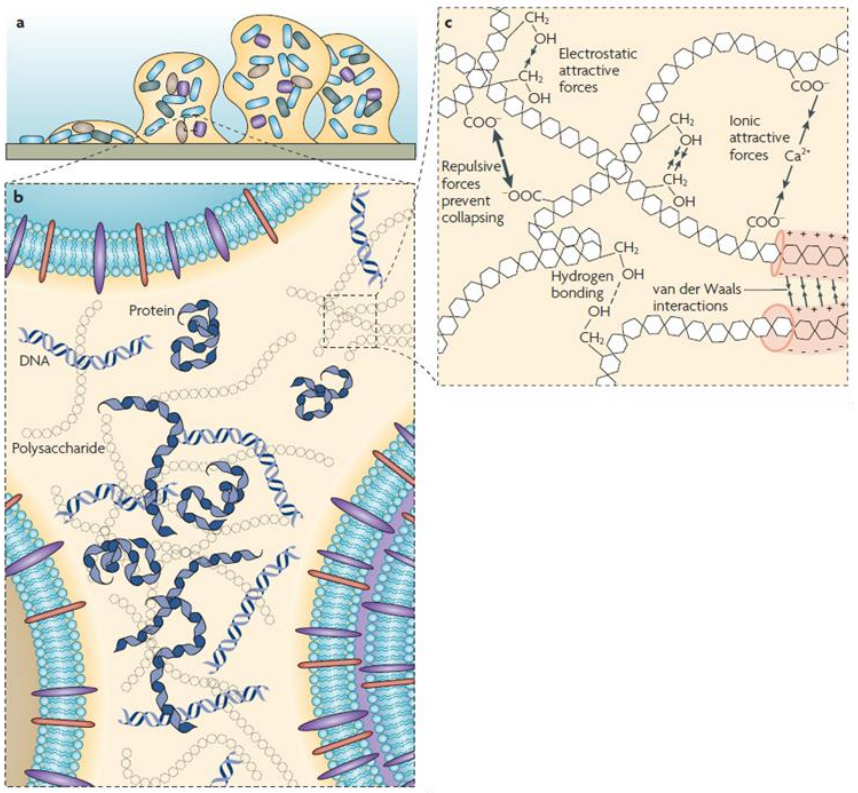


Figure 3 Schematic overview of the EPS matrix at different dimensions. a: Microorganisms imbedded in EPS attached to a surface. b: The main EPS matrix components between microbial cells. c: Different types of physiochemical interactions between EPS and the physical entanglement between the biopolymers which are providing the mechanical stability of the EPS matrix. Modified after Flemming & Wingender, 2010.[64]

Overall, biofilms represent a higher level of organization compared to single cells and offer inhabitants several emergent properties. Emergent properties are defined as new properties or structures of a system, resulting from the interaction of its elements. In terms of microorganisms, biofilms are providing resource capturing by sorption and retention, recycling of nutrients, nutrient transport through channel systems, an external digestion system by enzyme retention, the formation of synergistic micro-consortia, habitat diversity by localized gradients of oxygen, nutrient, pH, or quorum sensing molecules. Further they provide enhanced survival for their inhabitants when exposed to antimicrobials, metallic cations, and charged biocides, increased tolerance and resistance against environmental stressors such as protection against dehydration. Additionally, biofilms facilitate surface adhesion and horizontal gene transfer. These properties are the result of cell aggregations but would not occur in single cells.[64, 65, 67, 71-73]

1.10 Biofilm formation in the presence of petroleum

Due to their worldwide distribution, it is not surprising, that biofilms were found in connection with crude oil or bitumen, both naturally and in cultured in artificial medium. The formation of microbial mats by marine cyanobacteria in sea water was reported after massive exposure to crude oil.[74] Moreover, the crude oil degrading bacterium *Alcanivorax borkumensis* was found directly attached to the oil-water interface and detailed genome analysis showed the presence of genes related to biofilm formation.[24] Further, biofilm formation was observed on the surface of sand, clay, and rock particles in samples taken from a crude oil contaminated site in the Kuwaiti desert which was incubated in nutrient medium under aerobic conditions in the presence of crude oil.[75] Another study observed the formation of biofilms in crude oil containing mineral medium at aerobic conditions in well plates.[76]

Interestingly, in terms of bitumen, several former studies showed the formation of microbial biofilms directly on the bitumen surface after cultivation in bitumen containing nutrient medium under aerobic and anaerobic conditions, respectively. The produced CO_2 could directly be linked to biofilm development.[20, 28] Further, cultures originating from environmental samples of Alberta oil sand bitumen build up biofilms when cultivated under aerobic, microaerobic, and anaerobic growth conditions with and even without nutrient medium.[77] Some microorganisms even produce enhanced amounts of EPS with emulsifying properties in the presence of crude oil.[78]

These finding strongly support the assumption of biofilm formation as the prevalent form of life in deep subsurface petroleum reservoirs. Undisturbed microbial communities entrapped in tiny water droplets midst a bitumen phase could provide information on the microbial lifestyle and organization in petroleum reservoirs under natural conditions.

1.11 References – General introduction

1. Youssef, N.; Simpson, D.; Duncan, K.; McInerney, M.; Folmsbee, M.; Fincher, T.; Knapp, R., *In situ* biosurfactant production by *Bacillus* strains injected into a limestone petroleum reservoir. *Appl. Environ. Microbiol.* **2007,** *73*, 1239-1247.

2. Wolicka, D.; Borkowski, A., Micro-organisms and crude oil. *introduction to enhanced oil recovery (EOR) processes and bioremediation of oilcontaminated sites. L Romero-Zeron (Eds.). Croatia: Intech, Rijeka* **2012**, 113-142.

3. Youssef, N.; Elshahed, M. S.; McInerney, M. J., Microbial processes in oil fields: culprits, problems, and opportunities. *Adv. Appl. Microbiology.* **2009,** *66*, 141-251.

4. Mbadinga, S. M.; Wang, L.-Y.; Zhou, L.; Liu, J.-F.; Gu, J.-D.; Mu, B.-Z., Microbial communities involved in anaerobic degradation of alkanes. *Int. Biodeter. Biodegr.* **2011,** *65*, 1-13.

5. Zobell, C. E., The role of bacteria in the formation and transformation of petroleum hydrocarbons. *Science* **1945,** *102*, 364-369.

6. Bennett, B.; Adams, J.; Gray, N.; Sherry, A.; Oldenburg, T.; Huang, H.; Larter, S.; Head, I., 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.* **2013,** *56*, 94-105.

7. Larter, S.; Huang, H.; Adams, J.; Bennett, B.; Jokanola, O.; Oldenburg, T.; Jones, M.; Head, I.; Riediger, C.; Fowler, M., The controls on the composition of biodegraded oils in the deep subsurface: Part II—Geological controls on subsurface biodegradation fluxes and constraints on reservoir-fluid property prediction: Part I of this study was published in Organic Chemistry in 2003 (Larter et al., 2003). *Am. Assoc. Pet. Geol. Bull.* **2006,** *90*, 921-938.

8. Head, I. M.; Jones, D. M.; Larter, S. R., Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **2003,** *426*, 344.

9. Cai, M.; Jiménez, N.; Krüger, M.; Guo, H.; Jun, Y.; Straaten, N.; Richnow, H. H., Potential for aerobic and methanogenic oil biodegradation in a water flooded oil field (Dagang oil field). *Fuel* **2015,** *141*, 143-153.

10. Roadifer, R., Size Distributions of the World's Largest Known Oil and Tar Accumulations: Section I. Regional Resources. **1987**.

11. Bastin, E. S.; Greer, F. E.; Merritt, C. A.; Moulton, G., The presence of sulphate reducing bacteria in oil field waters. *Science* **1926,** *63*, 21-24.

12. Li, X.-X.; Mbadinga, S. M.; Liu, J.-F.; Zhou, L.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. *Int. Biodeter. Biodegr.* **2017,** *120*, 170-185.

13. Head, I.; Larter, S.; Gray, N.; Sherry, A.; Adams, J.; Aitken, C.; Jones, D.; Rowan, A.; Huang, H.; Röling, W., Hydrocarbon degradation in petroleum reservoirs. In *Handbook of hydrocarbon and lipid microbiology*, Springer: 2010; pp 3097-3109.

14. Magot, M.; Ollivier, B.; Patel, B. K. C., Microbiology of petroleum reservoirs. *Antonie Van Leeuwenhoek* **2000,** *77*, (2), 103-116.

15. Hallmann, C.; Schwark, L.; Grice, K., Community dynamics of anaerobic bacteria in deep petroleum reservoirs. *Nat. Geoscie.* **2008,** *1*, 588-591.

16. Kim, J. S.; Crowley, D. E., Microbial diversity in natural asphalts of the rancho la brea tar pits. *Appl. Environ. Microbiol.* **2007,** *73*, 4579-4591.

17. Vigneron, A.; Alsop, E. B.; Lomans, B. P.; Kyrpides, N. C.; Head, I. M.; Tsesmetzis, N., Succession in the petroleum reservoir microbiome through an oil field production lifecycle. *The ISME journal* **2017**.

18. Gao, P. K.; Tian, H. M.; Wang, Y. S.; Li, Y. S.; Li, Y.; Xie, J. X.; Zeng, B.; Zhou, J. F.; Li, G. Q.; Ma, T., Spatial isolation and environmental factors drive distinct bacterial and archaeal communities in different types of petroleum reservoirs in China. *Sci Rep-Uk* **2016,** *6*.

19. Wenger, L. M.; Davis, C. L.; Isaksen, G. H. In *Multiple controls on petroleum biodegradation and impact on oil quality*, SPE Annual Technical Conference and Exhibition, 2001; Society of Petroleum Engineers: 2001.

20. Ait-Langomazino, N.; Sellier, R.; Jouquet, G.; Trescinski, M., Microbial degradation of bitumen. *Experientia* **1991,** *47*, 533-539.

21. Aitken, C. M.; Jones, D. M.; Larter, S., Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature* **2004,** *431*, 291-294.

22. Bernard, F.; Connan, J.; Magot, M. In *Indigenous microorganisms in connate water of many oil fields: a new tool in exploration and production techniques*, SPE Annual Technical Conference and Exhibition, 1992; OnePetro: 1992.

23. Xiong, S.; Li, X.; Chen, J.; Zhao, L.; Zhang, H.; Zhang, X., Crude oil degradation by bacterial consortia under four different redox and temperature conditions. *Appl. Microbiol. Biotechnol.* **2015,** *99*, 1451-1461.

24. Schneiker, S.; dos Santos, V. A. P. M.; Bartels, D.; Bekel, T.; Brecht, M.; Buhrmester, J.; Chernikova, T. N.; Denaro, R.; Ferrer, M.; Gertler, C.; Goesmann, A.; Golyshina, O. V.; Kaminski, F.; Khachane, A. N.; Lang, S.; Linke, B.; McHardy, A. C.; Meyer, F.; Nechitaylo,

T.; Puhler, A.; Regenhardt, D.; Rupp, O.; Sabirova, J. S.; Selbitschka, W.; Yakimov, M. M.; Timmis, K. N.; Vorholter, F. J.; Weidner, S.; Kaiser, O.; Golyshin, P. N., Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nat. Biotechnol.* **2006,** *24*, 997-1004.

25. Abbasnezhad, H.; Gray, M.; Foght, J. M., Influence of adhesion on aerobic biodegradation and bioremediation of liquid hydrocarbons. *Appl. Microbiol. Biotechnol.* **2011,** *92*, 653-675.

26. Speight, J., Petroleum Asphaltenes-Part 1: Asphaltenes, resins and the structure of petroleum. *Oil Gas. Sci. Technol.* **2004,** *59*, 467-477.

27. Selucky, M. L.; Chu, Y.; Ruo, T.; Strausz, O. P., Chemical composition of Athabasca bitumen. *Fuel* **1977,** *56*, 369-381.

28. Wolf, M.; Bachofen, R., Microbial legradation of bitumen. *Experientia* **1991,** *47*, 542-548.

29. Chilingar, G. V.; Yen, T. F., *Bitumens, asphalts, and tar sands*. Elsevier Scientific Pub. Co. ;distributors for the U.S. and Canada, Elsevier North-Holland: Amsterdam ; New YorkNew York, 1978.

30. Kayukova, G.; Uspensky, B.; Abdrafikova, I.; Musin, R., Characteristic features of the hydrocarbon composition of Spiridonovskoe (Tatarstan) and Pitch Lake (Trinidad and Tobago) asphaltites. *Pet. Chem.* **2016,** *56*, 572-579.

31. Schulze-Makuch, D.; Haque, S.; Antonio, M. R. D.; Ali, D.; Hosein, R.; Song, Y. C.; Yang, J. S.; Zaikova, E.; Beckles, D. M.; Guinan, E.; Lehto, H. J.; Hallam, S. J., Microbial Life in a Liquid Asphalt Desert. *Astrobiology* **2011,** *11*, 241-258.

32. Stock, C., Rancho La Brea: a record of Pleistocene life in California. **1930**.

33. Clemente, J. S.; Fedorak, P. M., A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. *Chemosphere* **2005,** *60*, 585-600.

34. Meckenstock, R. U.; von Netzer, F.; Stumpp, C.; Lueders, T.; Himmelberg, A. M.; Hertkorn, N.; Schmitt-Kopplin, P.; Harir, M.; Hosein, R.; Haque, S., Water droplets in oil are microhabitats for microbial life. *Science* **2014,** *345*, 673-676.

35. Orphan, V.; Taylor, L.; Hafenbradl, D.; Delong, E., Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl. Environ. Microbiol.* **2000,** *66*, 700-711.

36. Mayumi, D.; Mochimaru, H.; Yoshioka, H.; Sakata, S.; Maeda, H.; Miyagawa, Y.; Ikarashi, M.; Takeuchi, M.; Kamagata, Y., Evidence for syntrophic acetate oxidation coupled

to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). *Environ. Microbiol.* **2011,** *13*, 1995-2006.

37. Lovley, D. R.; Chapelle, F. H., Deep subsurface microbial processes. *Rev. Geophys.* **1995,** *33*, 365-381.

38. Widdel, F.; Rabus, R., Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **2001,** *12*, 259-276.

39. Nazina, T. N.; Bidzhieva, S. K.; Grouzdev, D. S.; Sokolova, D. S.; Tourova, T. P.; Parshina, S. N.; Avtukh, A. N.; Poltaraus, A. B.; Talybly, A. K., *Soehngenia longivitae sp. nov*., a Fermenting Bacterium Isolated from a Petroleum Reservoir in Azerbaijan, and Emended Description of the Genus *Soehngenia*. *Microorganisms* **2020,** *8*, 1967.

40. Muyzer, G.; Stams, A. J., The ecology and biotechnology of sulphate-reducing bacteria. *Nat, Rev, Microbiol,* **2008,** *6*, 441-454.

41. Zengler, K.; Richnow, H. H.; Rosselló-Mora, R.; Michaelis, W.; Widdel, F., Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **1999,** *401*, 266.

42. Jones, D.; Head, I.; Gray, N.; Adams, J.; Rowan, A.; Aitken, C.; Bennett, B.; Huang, H.; Brown, A.; Bowler, B., Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **2008,** *451*, 176-180.

43. Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F., Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol. Rev.* **1998,** *22*, 459-473.

44. Xu, D.; Zhang, K.; Li, B.-G.; Mbadinga, S. M.; Zhou, L.; Liu, J.-F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Simulation of *in situ* oil reservoir conditions in a laboratory bioreactor testing for methanogenic conversion of crude oil and analysis of the microbial community. *Int. Biodeterior. Biodegradation.* **2019,** *136*, 24-33.

45. Liu, Y.-F.; Qi, Z.-Z.; Shou, L.-B.; Liu, J.-F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Anaerobic hydrocarbon degradation in candidate phylum 'Atribacteria'(JS1) inferred from genomics. *The ISME journal* **2019,** *13*, 2377-2390.

46. Savage, K. N.; Krumholz, L. R.; Gieg, L. M.; Parisi, V. A.; Suflita, J. M.; Allen, J.; Philp, R. P.; Elshahed, M. S., Biodegradation of low-molecular-weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates and community patterns. *FEMS Microbiol. Ecol.* **2010,** *72*, 485-495.

47. Chakraborty, R.; Coates, J., Anaerobic degradation of monoaromatic hydrocarbons. *Appl Microbiol Biotechnol* **2004,** *64*, 437-446.

48. Meckenstock, R. U.; Boll, M.; Mouttaki, H.; Koelschbach, J. S.; Tarouco, P. C.; Weyrauch, P.; Dong, X.; Himmelberg, A. M., Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. *J. Mol. Microbiol. Biotechnol.* **2016,** *26*, 92-118.

49. Aeckersberg, F.; Bak, F.; Widdel, F., Anaerobic oxidation of saturated hydrocarbons to CO 2 by a new type of sulfate-reducing bacterium. *Arch. Microbiol.* **1991,** *156*, 5-14.

50. Himmelberg, A. M.; Brüls, T.; Farmani, Z.; Weyrauch, P.; Barthel, G.; Schrader, W.; Meckenstock, R. U., Anaerobic degradation of phenanthrene by a sulfate-reducing enrichment culture. *Environ. Microbiol.* **2018,** *20*, 3589-3600.

51. Rueter, P.; Rabus, R.; Wilkest, H.; Aeckersberg, F.; Rainey, F. A.; Jannasch, H. W.; Widdel, F., Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature* **1994,** *372*, 455-458.

52. Jiménez, N.; Richnow, H. H.; Vogt, C.; Treude, T.; Krüger, M., Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *J. Mol. Microbiol. Biotechnol.* **2016,** *26*, 227-242.

53. Sun, X.; Kostka, J. E., Hydrocarbon-degrading microbial communities are site specific, and their activity is limited by synergies in temperature and nutrient availability in surface ocean waters. *Appl. Environ. Microbiol.* **2019,** *85*, e00443-19.

54. Johnsen, A. R.; Binning, P. J.; Aamand, J.; Badawi, N.; Rosenbom, A. E., The Gompertz function can coherently describe microbial mineralization of growth-sustaining pesticides. *Environ. Sci. Technology.* **2013,** *47*, 8508-8514.

55. Boschker, H.; Nold, S.; Wellsbury, P.; Bos, D.; De Graaf, W.; Pel, R.; Parkes, R. J.; Cappenberg, T., Direct linking of microbial populations to specific biogeochemical processes by 13 C-labelling of biomarkers. *Nature* **1998,** *392*, 801-805.

56. Niquette, P.; Servais, P.; Savoir, R., Bacterial dynamics in the drinking water distribution system of Brussels. *Water research* **2001,** *35*, 675-682.

57. Schulte, S.; Köster, D.; Jochmann, M.; Meckenstock, R., Applying reverse stable isotope labeling analysis by mid-infrared laser spectroscopy to monitor BDOC in recycled wastewater. *Sci. Total. Environ.* **2019,** *665*, 1064-1072.

58. Dong, X.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U., Quantification of microbial degradation activities in biological activated carbon filters by reverse stable isotope labelling. *AMB Express* **2019,** *9*, 109.

59. Dong, X.; Jochmann, M. A.; Elsner, M.; Meyer, A. H.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U., Monitoring microbial mineralization using

reverse stable isotope labeling analysis by mid-infrared laser spectroscopy. *Environ. Sci. Technol.* **2017,** *51*, 11876-11883.

60. Coplen, T. B., Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid. Commun. Mass. Spectrom.* **2011,** *25*, 2538-2560.

61. Jochmann, M. A.; Schmidt, T. C., *Compound-specific stable isotope analysis*. Royal Society of Chemistry: 2015.

62. Michener, R.; Lajtha, K., *Stable isotopes in ecology and environmental science*. John Wiley & Sons: 2008.

63. Stoodley, P.; Sauer, K.; Davies, D. G.; Costerton, J. W., Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **2002,** *56*, 187-209.

64. Flemming, H. C.; Wingender, J., The biofilm matrix. *Nat. Rev. Microbiol.* **2010,** *8*, 623-33.

65. Flemming, H.-C.; Wuertz, S., Bacteria and archaea on Earth and their abundance in biofilms. *Nat. Rev. Microbiol.* **2019,** *17*, 247-260.

66. Vert, M.; Doi, Y.; Hellwich, K. H.; Hess, M.; Hodge, P.; Kubisa, P.; Rinaudo, M.; Schue, F., Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). *Pure. Appl. Chem.* **2012,** *84*, 377-408.

67. Flemming, H.-C.; Baveye, P.; Neu, T. R.; Stoodley, P.; Szewzyk, U.; Wingender, J.; Wuertz, S., Who put the film in biofilm? The migration of a term from wastewater engineering to medicine and beyond. *NPJ Biofilms Microbiomes* **2021,** *7*, 1-5.

68. Persat, A.; Nadell, C. D.; Kim, M. K.; Ingremeau, F.; Siryaporn, A.; Drescher, K.; Wingreen, N. S.; Bassler, B. L.; Gitai, Z.; Stone, H. A., The Mechanical World of Bacteria. *Cell* **2015,** *161*, 988-997.

69. Sutherland, I. W., The biofilm matrix–an immobilized but dynamic microbial environment. *Trends Microbiol.* **2001,** *9*, 222-227.

70. Balzer, M.; Witt, N.; Flemming, H. C.; Wingender, J., Faecal indicator bacteria in river biofilms. *Water. Sci. Technol.* **2010,** *61*, 1105-1111.

71. Flemming, H. C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S. A.; Kjelleberg, S., Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **2016,** *14*, 563-575.

72. Flemming, H.-C., The perfect slime. *Colloids Surf B* **2011,** *86*, 251-259.

73. Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P., Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004,** *2*, 95-108.

74. Raghukumar, C.; Vipparty, V.; David, J. J.; Chandramohan, D., Degradation of crude oil by marine cyanobacteria. *Appl. Microbiol. Biotechnol.* **2001,** *57*, 433-436.

75. Obuekwe, C. O.; Al-Zarban, S. S., Bioremediation of crude oil pollution in the Kuwaiti desert: The role of adherent microorganisms. *Environ, Int,* **1998,** *24*, 823-834.

76. Mehdi, H.; Giti, E., Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. *Int. Biodeterior.* **2008,** *62*, 170-178.

77. Golby, S.; Ceri, H.; Gieg, L. M.; Chatterjee, I.; Marques, L. L.; Turner, R. J., Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. *FEMS Microbiol. Ecol.* **2012,** *79*, 240-250.

78. Calvo, C.; Martinez-Checa, F.; Toledo, F. L.; Porcel, J.; Quesada, E., Characteristics of bioemulsifiers synthesised in crude oil media by *Halomonas eurihalina* and their effectiveness in the isolation of bacteria able to grow in the presence of hydrocarbons. *App. Microbiol. Biotechnol.* **2002,** *60*, 347-351.

2 Aims of this study

The aim of this dissertation was to investigate and describe microbial life in recently discovered micro habitats dispersed through natural bitumen reservoirs. The following questions were examined to get more insights in microbial structures present in natural bitumen reservoirs:

- How common are these dispersed and inhabited water droplets in natural bitumen reservoirs? Are they a common feature of natural bitumen reservoirs or a unique phenomenon in Pitch Lake bitumen?
- How are these water droplets distributed in the bitumen phase?
- Can microorganisms be found in dispersed water droplets of other natural bitumen reservoirs?
- How many microorganisms are present in these micro habitats and are they alive and metabolically active?
- In case these microorganisms degrade bitumen, how active are they and what is their impact on the overall biodegradation process in natural bitumen reservoirs?
    - Can “reverse stable isotope labeling” be applied to measure the mineralization rates of natural bitumen?
- What kind of microorganisms are present in these droplets?
    - Are there similarities in the community composition within a bitumen reservoir and between different bitumen reservoirs?
- How are the present microorganisms organized? Are they living as planktonic individuals or arranged in biofilms?

3 Cumulative dissertation – Contribution statements

This cumulative dissertation comprises three articles Pannekens et al., 2019, Pannekens et al., 2020, and Pannekens et al., 2021. All three articles have been published in a peer-reviewed journals. In all three cases, Mark Pannekens has authored the manuscripts printed herein as a first author. Lisa Voskuhl (née Kroll) and Mark Pannekens are joint first authors in Pannekens et al., 2019.

3.1 Manuscript contribution statement - Oil reservoirs, an exceptional habitat for microorganisms

Reference: Pannekens, M.[1]; **Kroll, L.[1]**; Müller, H.; Mbow, F. T.; Meckenstock, R. U., Oil reservoirs, an exceptional habitat for microorganisms. *New Biotechnol* **2019**, 49, 1-9.

[1] These authors are contributed equally to this work and are regarded as joint first authors

DOI: https://doi.org/10.1016/j.nbt.2018.11.006

Statement of contributed work

Writing: Abstract, Chapter 3: Life in extreme environments – oil as an exceptional habitat, and conclusion.

Creating: Figure 1: Schematic scheme of a deep subsurface oil reservoir (oil leg) with underlying brine water (water leg) including figure description.

Completion: Text passages and references in the manuscript.

Correction: Incorporation of reviewer comments and composing the response letter.

3.2 Manuscript contribution statement - Densely Populated Water Droplets in Heavy-Oil Seeps

Declaration: The following manuscript Pannekens et al., 2020 was published in Applied and Environmental Microbiology, American Society for Microbiology. The usage permit is shown in the appendix (chapter 6.2.2). The manuscript is used as stated by the publisher without additional changes to the content.

Reference: Pannekens, M.; Voskuhl, L.; Meier, A.; Müller, H.; Haque, S.; Frösler, J.; Brauer, V. S.; Meckenstock, R.U., Densely populated water droplets in heavy oil seeps. *Appl Environ Microbiol* **2020**, 86, e00164-20.

DOI: https://doi.org/10.1128/AEM.00164-20

Statement of contributed work

Experimental contribution: Sampling campaign: Planning, equipment preparation, and shipping (Mark Pannekens). Sampling on field (Mark Pannekens, Rainer U. Meckenstock, Lisa Voskuhl, Huber Müller, Shirin Haque).

Microorganism visualization in water droplets: Method validation (Jan Frösler, Arne Meier under supervision of Mark Pannekens), Syto 9® *in situ* staining and CLSM analysis (Arne Meier under supervision of Mark Pannekens).

Droplet extraction: Extraction from bitumen samples (Mark Pannekens, Arne Meier, Lisa Voskuhl).

Cell counting: Light microscope counting (Arne Meier under supervision of Mark Pannekens), fluorescence microscope counting (Mark Pannekens).

LIFE/DEAD-Staining: Method validation SI (Mark Pannekens), Pitch Lake samples (Mark Pannekens), McKittrick and La Brea samples (Arne Meier under supervision of Mark Pannekens).

ATP analysis: ATP analysis of samples from Pitch Lake, McKittrick, and La Brea (Mark Pannekens).

Microbial community composition analysis based on 16S rRNA gene sequencing: Extraction, amplicon and index PCR, data analysis (Lisa Voskuhl). Extraction method validation (Verena S. Brauer).

Writing: Abstract, importance paragraph, introduction, results, discussion, supplemental material (Mark Pannekens)

Material and Methods: Oil sampling, droplet *in situ* observations, droplet sampling, cell counting in individual droplets, determination of cell membrane integrity in individual droplets, ATP quantification (Mark Pannekens), DNA extraction, 16S rRNA gene amplification, library preparation, and sequencing (Lisa Voskuhl).

Figures and Tables: Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, (Mark Pannekens), Figure 6 and Figure S1 (Lisa Voskuhl), table 1 (Lisa Voskuhl and Mark Pannekens), table S1 (Mark Pannekens).

3.3 Manuscript contribution statement - Microbial degradation rates of natural bitumen

Pannekens, M.; Voskuhl, L.; Mohammadian, S.; Köster, D.; Meier, A.; Köhne, J. M.; Kulbatzki, M.; Akbari, A.; Haque, S.; Meckenstock, R. U., Microbial degradation rates of natural bitumen. *Environ Sci Technol* **2021,** 55, 8700-8708.

DOI: https://pubs.acs.org/doi/10.1021/acs.est.1c00596

Statement of contributed work

Experimental contribution: Sampling campaign: Planning, equipment preparation, and shipping (Mark Pannekens). Sampling on field (Mark Pannekens, Rainer U. Meckenstock, Anne Himmelberg, Shirin Haque).

Geochemistry determination: Anion and cation measurements and data evaluation (Mark Pannekens).

Artificial saltwater medium and experimental setup: Testing, preparation, and microcosm setup (Mark Pannekens).

Bitumen isotope amount fraction $x(^{13}C)$: Sample preparation (Mark Pannekens), measurement and data evaluation (Daniel Köster).

Bitumen density: sample preparation and measurement (Mark Pannekens).

Sulfate determination: Sulfate measurements and data analysis (Mark Pannekens).

Bitumen mineralization to carbon dioxide: ratio measurement $^{12}C/^{13}C$-CO_2 via delta ray and data evaluation (Mark Pannekens).

Cell counting: Cell counting via Thoma Chamber and light microscope (Mark Pannekens).

Microbial community composition based on 16S rRNA gene sequencing: DNA extraction (Mark Pannekens, assisted by Michelle Kulbatzki), extract purification (Mark Pannekens), amplicon PCR (Mark Pannekens, assisted by Michelle Kulbatzki), index PCR (Lisa Voskuhl, Mark Pannekens), data evaluation (Lisa Voskuhl).

Biofilm formation on bitumen surface: Method validation, drinking water biofilm controls (Arne Meier under supervision of Mark Pannekens), sample preparation, lectin staining, CLSM pictures (Mark Pannekens).

Water content and distribution of droplets in the bitumen: column design (Mark Pannekens), bitumen sampling (Lisa Voskuhl, Rainer Meckenstock), column scans (Sadjad Mohammadian, John. M. Köhne), data evaluation (Sadjad Mohammadian, Mark Pannekens).

Calculations: Water content, surface area, electron balance (Mark Pannekens).

Writing: Abstract, introduction, results and discussion (Mark Pannekens).

Material and Methods: Bitumen sampling, microcosm setup, assessing bitumen mineralization, microbial community composition, biofilm visualization on bitumen surface (Mark Pannekens), 3D imaging of bitumen – droplet distribution (Sadjad Mohammadian, Mark Pannekens).
Supporting information: Geochemistry of water extracted from the bitumen body, biofilm visualization on bitumen surface, Syto9-lectin tests on drinking water biofilms, sulfate measurements during the experiment (Mark Pannekens), DNA extraction, 16S rRNA gene amplification, library preparation and sequencing (Lisa Voskuhl, Mark Pannekens), 3D imaging of bitumen – droplet distribution (Sadjad Mohammadian, Mark Pannekens).

Figures and tables: Graphical abstract (Mark Pannekens), figure 1, figure 3, figure S1 (Mark Pannekens), figure 2 (Lisa Voskuhl), and figure 4 (Sadjad Mohammadian). Tables S1 and table S2 (Mark Pannekens).

4 Publications

4.1 Oil reservoirs, an exceptional habitat for microorganisms

Mark Pannekens[1], Lisa Kroll[1], Hubert Müller, Fatou Tall Mbow and Rainer U. Meckenstock*

University of Duisburg-Essen, Biofilm Centre, Universitätsstr. 5, 41451 Essen, Germany

ARTICLEINFO

Keywords:

Oil reservoir; Microbial ecology; Biodegradation; Oil-water transition zone; Biofilm; Virus

Abbreviations: OWTZ, oil-water transition zone; EPS, extracellular polymeric substances; PAH, polycyclic aromatic hydrocarbons; EOR, enhanced oil recovery; OTUs, operational taxonomic units; SRB, sulfate-reducing bacteria

* Corresponding author at: University of Duisburg-Essen, Biofilm Centre, Universitätsstr. 5, 41451, Essen, Germany.

E-mail address: rainer.meckenstock@uni-due.de (R.U. Meckenstock).

[1] These authors are contributed equally to this work and are regarded as joint first authors.

https://doi.org/10.1016/j.nbt.2018.11.006

Available online 28 November 2018

ABSTRACT

Microorganisms are present in oil reservoirs around the world where they degrade oil and lead to changes in oil quality. Unfortunately, our knowledge about processes in deep oil reservoirs is limited due to the lack of undisturbed samples. In this review, we discuss the distribution of microorganisms at the oil-water transition zone as well as in water saturated parts of the oil leg and their possible physiological adaptations to abiotic and biotic ecological factors such as temperature, salinity and viruses. We show the importance of studying the water phase within the oil, because small water inclusions and pockets within the oil leg provide an exceptional habitat for microorganisms within a natural oil reservoir and concurrently enlarge the zone of oil biodegradation. Environmental factors such as temperature and salinity control oil biodegradation. Temperature determines the type of microorganisms which are able to inhabit the reservoir. *Proteobacteria* and *Euryarchaeota*, are ubiquitous in oil reservoirs over all temperature ranges, whereas some others are tied to specific temperatures. It is proposed that biofilm formation is the dominant way of life within oil reservoirs, enhancing nutrient uptake, syntrophic interactions and protection against environmental stress. Literature shows that viruses are abundant in oil reservoirs and the possible impact on microbial community composition due to control of microbial activity and function is discussed.

4.1.1 Introduction

Oil reservoirs are extreme environments for microbial life [1] characterized by high toxicity, hydrophobicity and low water activity, as well as high temperature, salinity, and pressure [2]. Nevertheless, oil reservoirs offer a broad range of niches for a multitude of bacteria and archaea, such as sulfate-, nitrate-, and iron-reducers, fermenters, acetogens, and methanogens [1, 3]. The microbial degradation of oil results in a higher fraction of bitumen and eventually leads to the deterioration of the world's oil resources. Since oil is still one of the most important resources for industry and energy [4], it is crucial to gain insights into the microbiology of oil reservoirs. Over the past decades, numerous reviews on oil microbiology have investigated the extent of biodegradation, the effect of microbes on oil quality, oil production methods and enhanced oil recovery (EOR) [3, 5-8], as well as their taxonomical and functional composition and the impact of environmental factors on microbes [3, 7, 9, 10]. However, due to the lack of undisturbed samples, our knowledge of microbial ecology in oil reservoirs is still limited [11].

4.1.2 Distribution of microbes within oil reservoirs

Oil reservoirs consist of different phases where microorganisms can thrive, such as crude oil, formation water and solid surfaces from rock and organic materials [12]. To understand the oil-water distribution patterns of microbes, it is important to conceptualize the oil habitat. In general, microbial degradation of oil is limited by the availability of electron acceptors because, due to thermodynamic constraints, hydrocarbons cannot be fermented without a hydrogen and acetate scavenging process. However, microbes can only conserve energy if they have direct contact to both electron donors from the oil phase and electron acceptors from the water phase [13, 14]. This situation is given at the oil-water transition zone (OWTZ) beneath the oil leg, which is a hotspot of microbial growth and oil degradation [5, 15] (Fig 1). Here, the oil phase provides electron donors and the water phase provides the habitat for the microorganisms. Consequently, the rate of oil biodegradation depends strongly on the size of the surface of the oil-water interface.

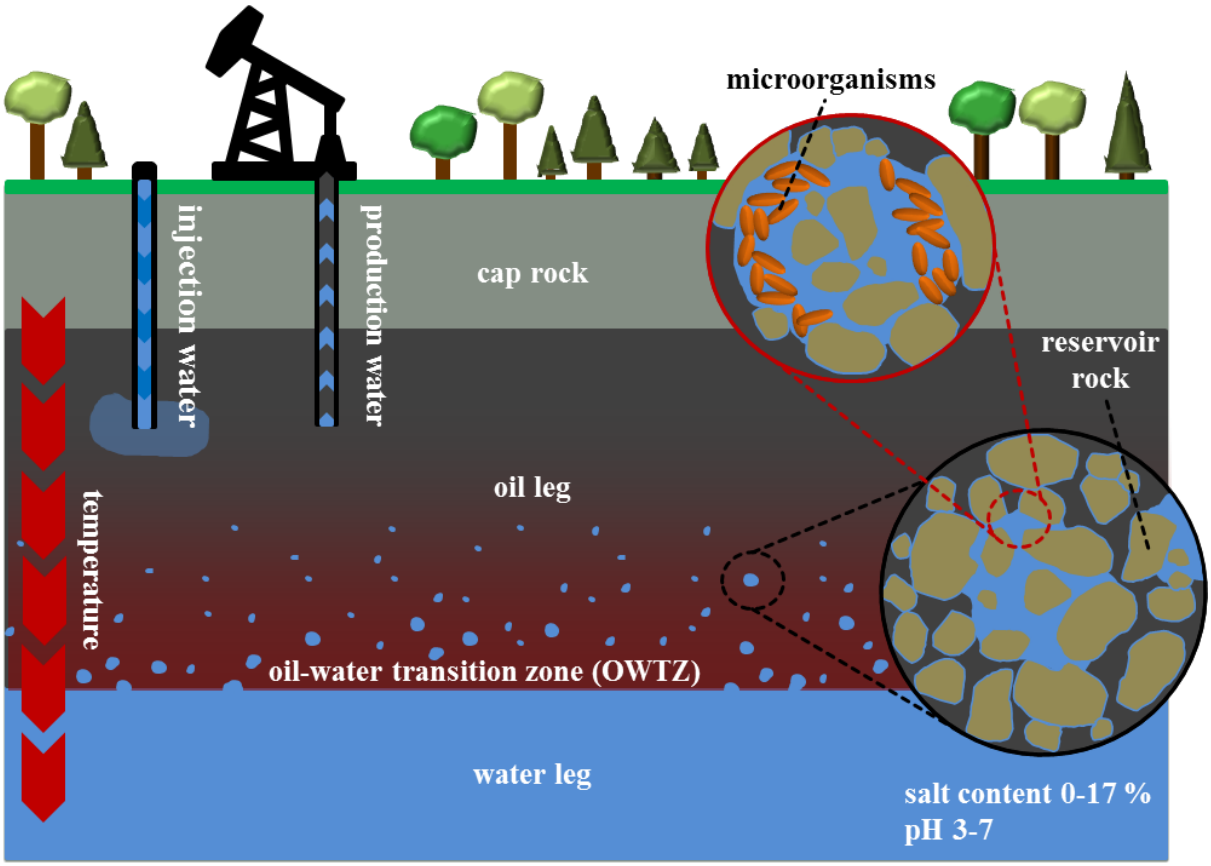


Fig. 1. Schematic scheme of a deep subsurface oil reservoir (oil leg) with underlying brine water (water leg). Most of the biological oil degradation takes place at the oil-water transition zone (OWTZ) and in dispensed water droplets nearby. Microorganisms live attached to rock particles in a thin water film or in dispensed water droplets amidst the oil phase.

In deep oil reservoirs, dissolved electron acceptors oxygen and nitrate are naturally absent unless anthropogenically added via injected fluids [8]. Several studies detected iron-, manganese-, or nitrate-reducing bacteria such as *Shewanella putrefaciens* or *Deferibacter thermophilus* in fluids of oil reservoirs [16-20]. However, solid iron(III) and manganese(IV) oxides typically are not available as electron acceptors for microbial oil degradation because they have been reduced over the millions of years and are not replenished. Therefore, the most prevalent processes are methanogenesis and sulfate reduction, if there is a source of sulfate [8, 21, 22].

It has been a paradigm for the last decades that biodegradation mostly takes place directly near the OWTZ. However, studies have shown an increasing saturated hydrocarbon content over approximately 100 to 130 meters away from the OWTZ due to circulation and diffusion [5]. Nevertheless, there is always a certain amount of water also present in the oil leg either as water-saturated areas in the pore space of the rock or as a thin water film covering the rock surface in water wet reservoirs. Thus, different oil reservoirs vary in water content as well as in their oil composition [5, 7, 23, 24]. Interestingly, oil samples with high water content of around 40-60% harbor a 2.6-fold higher bacterial richness compared to low water content oils with 1-5% [25] indicating that the amount of water present in the oil leg plays an important role as a habitat for the microorganisms.

In fact, stratified water pockets and pore spaces were discovered in the natural asphalts from the Rancho La Brea Tar Pits in Los Angeles, CA [26], and small water inclusions were discovered in the oil phase of the Pitch Lake in Trinidad and Tobago, the world largest natural tar lake [27]. The 1-3 µl water droplets from Trinidad and Tobago were densely populated with complex microbial communities and actively degrading the oil. The high salinity and water-stable isotope measurements indicated that the water droplets originated from deep subsurface formation water, most likely directly form the oil reservoir feeding the natural oil seep through a geological fracture [27]. Taking the water droplets as a proxy for subsurface processes, it is very likely that significant microbial populations can thrive within water-filled rock pores away from the OWTZ. Consequently, microbial degradation potential in the reservoir should correlate with the water content of the different phases, building a gradient of degradation activity starting from the OWTZ at the bottom and decreasing to the top of the reservoir (Fig. 1). In fact, such patterns of biodegraded oil can be found in reservoirs although they have been interpreted as diffusion gradients of alkanes from the non-degraded oil at the top of the reservoirs towards the depletion hot spot at the OWTZ [11]. Nevertheless, the findings from Tar Pits in Los Angeles and of the dispersed water droplets in the natural oil

seep in Trinidad indicate that microbes reside in water pockets within the oil phase or even in the water film around sand grains [26, 27]. This concept enlarges the overall oil-water interface and should be considered as having a large impact on the degradation process. Microbial oil biodegradation can consequently not only occur at the oil-water transition zone but also within the oil leg (Fig. 1).

Upon production, oil is pumped to the surface as an artificial mixture of water, oil and gas. One has to be aware that this mixture does not necessarily contain the true composition of the microbial communities in the subsurface. Most of the microbes that thrive either at the OWTZ or in the water-filled compartments in the oil leg will thrive attached to the rock matrix rather than planktonic in the water phase and will not appear in oil or produced water. Furthermore, cells not attached to the rock are probably present in dense biofilms at the oil-water interface.

Most studies on oil microbiomes classify samples from oil wells as either oil or water phase. The natural water phase mostly consists of formation water, which is naturally present within porous rock as explained above [3, 28]. The amount and composition of formation water, its natural flow-rate and its flow-path through the oil field can influence the microbial community [3, 24, 29]. Most studies assume that DNA extracted from the formation water represents the microbiome of the total reservoir [28, 30, 31]. However, analysis of water- and oil phases have revealed vast differences between their microbial community compositions [13]. The bacterial diversity in the oil phase [28] and species richness [12] appeared to be much higher than those of the water phase. Some bacteria might attach directly to the oil surface, while others remain in the water phase. *Acidobacteria*, *Actinobacteria*, *Fusobacteria*, *Nitrospira*, *Pseudomonadales*, and *Thermodesulfobacteria* are more frequently isolated from the oil phase, whereas *Alphaproteobacteria*, *Atribacteria*, *Bacteriodetes*, *Betaproteobacteria*, *Campylobacterales*, *Chloroflexi*, *Synergistetes*, and *Thermotogae* are mostly isolated from the water phase [9, 28].

Thus, the water phase itself contains only a minor portion of the microorganisms in the oil reservoir and can only constitute a proxy for the true community composition. The oil phase contains another part of the microbial communities, most likely containing attached microbes and those present in small water droplets dispersed in the oil [9, 27]. Thus, at least the two phases, water and oil, should be analyzed to obtain a better picture of the true microbial community composition.

4.1.3 Life in extreme environments – oil as an exceptional habitat

Biofilms as a physiological adaptation to life in oil?

Microbial life in oil reservoirs faces severe conditions with multiple stressors such as toxicity of the oil and low water activity. In addition, planktonic microorganisms often do not have access to both electron donor and electron acceptor as a prerequisite for microbial energy conservation and must attach to the oil-water interface. One microbial adaption to these harsh conditions is organization in biofilms. Microbial biofilms are widely distributed in nature and belong to the most successful strategies of life on earth [32]. The formation of biofilms was shown in the presence of crude oil under aerobic conditions such as in an oil lake in the Kuwaiti desert [33] or as the formation of microbial mats by cyanobacteria in seawater [34, 35]. Generally, biofilms are defined as 'aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface' [36]. EPS are a gel-like network that keep the microbial aggregates together in order to provide mechanical stability to biofilms in flowing or moving systems such as water and oil [37-41]. The sticky coating of a biofilm matrix is a protective layer against external environmental stressors including desiccation, temperature and salt concentrations in oil fields. In the presence of crude oil, many bacteria produce EPS, which act as biosurfactants to enhance the solubility and bioavailability of hydrophobic organic compounds, such as polycyclic aromatic hydrocarbons (PAH) or n-alkanes [42-48]. In addition, a biofilm provides space for mutualistic micro-consortia, confined gradients increasing habitat diversity, resource capture by nutrient sorption, nutrient transport by channel systems, exchange of signaling molecules and acts as a barrier against toxic compounds by increased tolerance due to horizontal gene transfer [49-52]. Because of the protective and nutrient transport properties of biofilms, it is not surprising that they have been found at the alkane-water interface of n-alkanes (C_8-C_{28}) and n-alcohols (C_{12} and C_{16}) [45, 47, 53]. Some enrichment cultures were able to build biofilms on the surface of phenanthrene and other PAHs to overcome the mass transfer limitations during the degradation [44, 46]. Biofilm formation also depends on the solubility of the PAHs; lower solubility results in more attached cells and biofilm formation to overcome the mass transfer limitations [46]. Due to these advantages of biofilms in toxic and extreme environments, we propose that biofilm formation is also the predominant form of life in oil reservoirs.

Metabolic functions and interactions of microorganisms in oil reservoirs

In the absence of the most favorable electron acceptors oxygen and nitrate, sulfate reduction and syntrophic methanogenesis are the dominant processes in oil biodegradation [3]. If sulfate is present at concentrations higher than 50 µM, hydrocarbon degradation coupled to sulfate reduction is the dominating process over methanogenesis [54]. Sulfate-reducing microorganisms are phylogenetically diverse and can be found within the *Proteobacteria, Firmicutes, Nitrospira* and *Thermodesulfobacteria*, as well as in the *Crenarchaeota* and *Euryarchaeota* [55]. If sulfate is absent as electron acceptor, many sulfate-reducers can switch their metabolism to fermentative oil degradation, producing short chain fatty acids, molecular hydrogen and carbon dioxide [56, 57]. For example, members of the genera *Desulfovibrio*, *Desulfotomaculum* and *Archaeoglobus* can grow with sulfate as electron acceptor or as fermenters in association with methanogens when sulfate is depleted [56, 58-61]. Fermentation of hydrocarbons is thermodynamically only feasible when coupled to methanogenesis depleting both hydrogen and acetate [22, 59, 62-68]. Thus, methanogenic oil degradation is always a syntrophic process, where different members of the microbial community perform different steps in an overall metabolic process which cannot be fulfilled by a single member alone [69]. In addition, methane can be generated by acetoclastic methanogens disproportioning acetate to CO_2 and methane or by methylotrophic methanogenesis [70]. In fact, most of the biological methane generated in oil reservoirs originates from acetoclastic methanogenesis as indicated by stable isotope signatures [71]. Syntrophic interactions in oil reservoirs are not confined to a specific phylogenetic group of prokaryotes [56, 59, 63, 72]. The two bacterial phyla *Proteobacteria* and *Firmicutes*, and the three classes *Archaeoglobi*, *Methanomicrobia* and *Methanobacteria* affiliated to *Euryarchaeota*, are mostly involved in syntrophic interactions (Table 1) [56, 63, 72-75].

Table 1 Phylogenetic affiliation of syntrophic consortia in oil reservoirs and their metabolic potentials.

phylum / class	order / genus	metabolic capacities	references
Proteobacteria / Gammaproteobacteria	*Alteromonadales / Marinobacter*	syntrophic alkane degraders	[63]
Proteobacteria / Deltaproteobacteria	*Syntrophobacterales / Smithella*		

Proteobacteria / Deltaproteobacteria	*Syntrophobacterales / Syntrophus*	syntrophic fatty acids and alkanes degraders in association with methanogens	[64]
Proteobacteria / Deltaproteobacteria	*Desulfovibrionales / Desulfovibrio*	metabolically versatile, sulfate respiration and syntrophic alkane fermenters in association with methanogens	[56, 60]
Firmicutes / Clostridia	*Clostridiales / Desulfotomaculum*		[56, 61]
Firmicutes / Clostridia	*Clostridiales / Clostridium*		[56, 73]
Euryarchaeota / Archaeoglobi	*Archaeoglobales / Archaeoglobus*	sulfate-reducing archaea and syntrophic fermentative alkanes degrader in association with acetotrophic methanogens	[59, 72]
Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanoculleus*	hydrogenotrophic methanogens	[56, 73]
Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanosaeta*	acetotrophic methanogens	[56, 73]
Euryarchaeota / Methanobacteria	*Methanobacteriales / Methanobacterium*	hydrogenotrophic methanogens	[72]

Many *Proteobacteria* are known to be syntrophic alkane degraders, e.g. members of the genera *Marinobacter* and *Smithella* have been highly enriched in methanogenic oil-degrading cultures [63, 68, 72, 73, 76]. Members of the genus *Syntrophus* can degrade alkanes and fatty acids in syntrophic association with methanogens [64, 72]. As methanogenic and fermentative microorganisms are strongly dependent on each other, they are frequently organized in close vicinity to each other in order to provide a rapid exchange of electrons by diffusion of hydrogen or formate [67]. Furthermore, an electron exchange by direct interspecies electron transfer has been discovered; *Geobacter metallireducens* transfers electrons directly to *Metanosaeta harundinacea* during methanogenic degradation of ethanol, presumably by

nanowires [77, 78]. In addition, inorganic, electrically conductive particles inside a biofilm matrix can support interspecies electron transfer [79]. Thus, we suggest that the methanogenic degradation of oil mainly takes place in mutualistic microbial consortia organized in biofilms, where fermenting microbes transfer electrons either directly or indirectly to the methanogens.

Anthropogenic impacts – injection water

Oil production is the major anthropogenic factor influencing microbial communities in oil reservoirs. This includes drilling, flooding, hot steam and water injections, all of which lead to a high potential of invasion of external microorganisms into the original microbial communities [9, 24, 29]. Water injections are necessary in secondary oil production stages to increase reservoir pressure. The amount of injected water depends on the reservoir pressure, well age or water progressing within the reservoir. Oil companies use different types of injection waters consisting of either seawater, fresh water or recycled formation water. Offshore fields are mostly supplied with seawater [24, 80] whereas in other oil fields groundwater [81] or surface water [30] are used. In some reservoirs, injection waters are enriched with chemicals or nutrients in order to manipulate the indigenous microbial community. For instance, nitrate and nitrite injections are used to suppress reservoir souring by microbial H_2S production. Oxygen injections can stimulate the aerobic hydrocarbon metabolism and mobilize the oil within the well by lowering the interfacial tension between oil and water phase through biosurfactant producing microbes or changes in the oil matrix [82]. Alternatively, fermentative bacteria and carbohydrate injection lead to the generation of acids, gases and solvents, which increases oil output, so called enhanced oil recovery (EOR) [8, 31]. Water injections decrease the temperature of the oil field and build up a temperature gradient. The injection volume also affects the chemical composition of the production water, as it decreases the concentrations of magnesium, potassium, nitrate, nitrite and sulfate [24]. Production water is a byproduct of oil production and has been transported through the oil phase and pumped to the surface; it can be a mixture of formation and injection water and can contain particles and soluble compounds from oil [11, 83] (Fig. 1). Two comparative studies have demonstrated higher concentrations of ammonium and fatty acids in formation water compared to production water [24]. Produced fluids with less than 10% injection water content did not have significant influence on microbial composition and metabolic potential. In contrast, fluids with a higher injection water cut indicated that the community composition and metabolic potential can be altered by the water composition. A close correlation was calculated between the relative abundance of the genus *Flexistipes*, family *Deferribacteres*,

and the proportion of injected seawater and the concentrations of magnesium, potassium, nitrate, nitrite, and sulfate. *Epsilonproteobacteria* and *Gammaproteobacteria* were isolated in greater abundance from sample wells with the highest water injection rate [24]. Several studies have reported a relationship between the chemical composition of the oil reservoirs and the operational taxonomic units (OTUs) found therein. In Algerian oil reservoirs, production waters revealed significant correlations between the relative abundance of bacterial OTUs or phyla and Cl^- and K^+ ions. However, it is not clear if this correlation is causative. It may well be that the real causes for microbial community differences are for example differences in water content and structure of the reservoir and that the ion composition is just an indication of geological differences. Significant differences between microbial community composition of production and injection waters were observed for waters from Algerian oilfields and the offshore Halfdan oil field in the Danish North Sea. The Algerian oilfield injection water was richer in cells and dominated by bacteria, whereas the production water contained ten times fewer cells and was dominated by Archaea [24, 81]. This difference indicates a trivial correlation between oil degradation processes in the methanogenesis-dominated reservoir and the microbial community composition in the production water.

Oil quality is determined by the degree of biodegradation and physical processes during oil production such as water injection or phase fractionation explained above. Those processes lead to lower concentrations of specific isomers, hydrocarbons, sulfur-, oxygen- and nitrogen-containing compounds [84, 85] and an increase in oil viscosity, metals and microbial metabolites such as organic acids or sulfur compounds. Biodegraded oil reservoirs typically consist of oil-water emulsified fluids and systematic gradients built by different oil components [8, 24]. For instance, isoprenoids and n-alkanes concentration decrease towards the OWTZ as they are degraded faster than aromatic compounds [11, 86]. There, the degradation of oil is also controlled by the nutrient availability in the individual reservoir [8]. In contrast to many oil reservoirs, the bulk water contained in oil of the Pitch Lake in Trinidad and Tobago revealed that concentrations of essential nutrients, such as 95 mg/L ammonia and 5 mg/L phosphate, were not growth-limiting [27] demonstrating again that conditions within the oil phase allow biodegradation. [87]

Abiotic factors – temperature, pH, and salinity

The geology of an oil reservoir determines the temperature, pH and salinity, which influence the composition and metabolic activity of the indigenous microbiota. Temperature is one of the most important factors determining microbial community composition in oil reservoirs [1, 3, 8-10, 24, 29, 81]. Temperature increases by about 2 to 3 °C per 100 meter of depth, which means that the effects of depth and temperature are closely related [3, 81]. Generally, temperature is higher in reservoirs during primary production before injection, compared to similar reservoirs where water injections cool down the reservoir during secondary production [9, 24]. The maximum temperature for hydrocarbon degradation in oil reservoirs is generally accepted to be around 82 °C [3, 88]. The extreme solvent stress of the oil increases with elevated temperature and most likely the integrity of the cell membranes suffers. A study detected hyperthermophilic microorganisms in reservoirs with well temperatures up to 131 °C [80]. However, as the real conditions in the habitat could not be determined, it is highly unlikely that the organisms really thrived at that temperature *in situ*. So far, the record in hyperthermophilic growth is at 95 °C by the bacterium *Aquifex pyrophilus* and at 113 °C by the archaeon *Pyrolobus fumarii*, which of course did not take place in the presence of hydrocarbons [89]. Therefore, the detection of life at 131 °C is questioned by the indirect estimation of the oil temperatures and the so far known temperature maxima of microbes [10, 80, 81, 90].

Nevertheless, highest microbial diversity has been found at moderately hot reservoirs with temperature of around 55 °C [91]. As everywhere in the environment, oil reservoirs harbor microorganisms with different temperature preferences (Table 2**Table**).

Table 2 Bacteria and archaea typically associated with low-temperature (< 50 °C) or high-temperature (≥ 50 °C) petroleum reservoirs.

temperature optimum	**phylum / class**	**order / genus**	**reference**
ubiquitous	*Proteobacteria / Epsilonproteobacteria*	*Campylobacterales / Arcobacter*	[29]
	Proteobacteria / Epsilonproteobacteria	*Campylobacterales / Sulfurospirillum*	
	Proteobacteria / Gammaproteobacteria	*Pseudomonadales / Pseudomonas*	
	Proteobacteria / Alphaproteobacteria	*Rhizobiales / Rhizobium*	
	Proteobacteria / Alphaproteobacteria	*Sphingomonadales / Sphingomonas*	
	Acinetobacter		
only > 50 °C	*Crenarchaeota / Thermoprotei*	*Fervidicoccales*	[9]
	Euryarchaeota / Halobacteria	*Halobacteriales*	

	Euryarchaeota / Halobacteria	*Haloferacales*	
	Thaumarchaeota / Nitrososphaeria	*Nitrososphaerales*	
	Nitrospirae / Nitrospira	*Nitrospirales / Nitrospira*	
	Crenarchaeota / Thermoprotei	*Sulfolobales*	
	Proteobacteria / Deltaproteobacteria	*Syntrophobacterales / Thermosulforhabdus*	[93]
	Euryarchaeota / Thermoplasmata	*Thermoplasmatales*	[9, 31]
	Crenarchaeota / Thermoprotei	*Thermoproteales*	[9]
	Acidobacteria		[9, 29]
	Atribacteria		[9]
mostly > 50 °C	*Euryarchaeota / Archaeoglobi*	*Archaeoglobales*	[8, 9, 29]
	Firmicutes / Bacilli	*Bacillales / Anaerobacillus*	[29]
	Firmicutes / Bacilli	*Bacillales / Bacillus*	
	Firmicutes / Clostridia	*Clostridiales / Thermosyntropha*	
	Euryarchaeota / Halobacteria	*Halobacteriales / Halogeometricum*	[31]
	Proteobacteria / Hydrogenophilalia	*Hydrogenophilales / Tepidiphilus*	[29]
	Thermotogae / Thermotogae	*Kosmotoga*	
	Euryarchaeota / Methanobacteria	*Methanobacteriales / Methanothermobacter*	[29, 31, 94, 95]
	Euryarchaeota / Methanomicrobia	*Methanocellales / Methanocella*	[31]
	Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanocalculus*	[29]
	Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanosaeta*	[29, 31]
	Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanomethylovorans*	[31]
	Nitrospirae / Nitrospira	*Nitrospirales / Thermodesulfovibrio*	[29]
	Proteobacteria / Alphaproteobacteria	*Rhodospirillales / Tistrella*	
	Deinococcus–Thermus / Deinococci	*Thermales / Thermus*	
	Firmicutes / Clostridia	*Thermoanaerobacterales / Thermoanaerobacter*	[8, 10]
	Euryarchaeota / Thermococci	*Thermococcales*	[8, 9, 24, 29, 31]
	Euryarchaeota / Thermoplasmata	*Thermoplasmatales / Thermogymnomonas*	[31]
	Actinobacteria / Thermoleophilia		[29]
	Bacteroidia / Bacteroidia		
	Deferribacteres / Deferribacteres		[24]
	Firmicutes		[31]
	Proteobacteria / Betaproteobacteria		[29]
	Proteobacteria / Deltaproteobacteria		

	Tenericutes / Mollicutes		
	Thermodesulfobacteria		[31]
	Thermotogae		
mostly < 50 °C	*Actinobacteria / Actinobacteria*	*Actinomycetales / Microbacterium*	[29]
	Actinobacteria / Actinobacteria	*Actinomycetales / Dietzia*	
	Actinobacteria / Actinobacteria	*Actinomycetales / Rhodococcus*	
	Proteobacteria / Gammaproteobacteria	*Alteromonadales / Marinobacterium*	
	Crenarchaeota / Thermoprotei	*Desulfurococcales*	[9]
	Euryarchaeota / Methanobacteria	*Methanobacteriales / Methanobacterium*	[31, 95, 96]
	Euryarchaeota / Methanomicrobia	*Methanocellales*	[9]
	Euryarchaeota / Methanococci	*Methanococcales / Methanococcus*	[29]
	Euryarchaeota / Methanomicrobia	*Methanomicrobiales*	[9, 95, 96]
	Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanocorpusculum*	[29]
	Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanoculleus*	[29, 31]
	Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanolinea*	[29]
	Euryarchaeota / Methanomicrobia	*Methanosarcinales*	[9]
	Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanolobus*	[29]
	Proteobacteria / Alphaproteobacteria	*Rhodobacterales / Donghicola*	
	Proteobacteria / Alphaproteobacteria	*Rhodobacterales / Hyphomonas*	
	Proteobacteria / Alphaproteobacteria	*Rhodobacterales / Paracoccus*	
	Bacteroidetes		[31, 97]
	Chloroflexi		[9]
	Planctomycetes		
	Proteobacteria		[31]
	Spirochaetes		[9]
	Synergistetes		

Nitrospira, *Atribacteria* and *Acidobacteria* were only detected in high-temperature oil reservoirs above 50 °C. Most *Gammaproteobacteria*, like *Firmicutes*, *Thermotogae* and *Thermodesulfobacteria*, showed a higher relative abundance in high-temperature oil reservoirs above 50 °C. *Spirochaetes*, *Synergistetes*, *Chloroflexi*, *Marinobacterium*, *Paracoccus*, *Donghicola* and *Planctomycetes* were more frequently detected in oil reservoirs below 50 °C. The archaea *Haloferacales*, *Thermoproteales*, *Sulfolobales*, *Nitrososphaerales*, *Halobacteriales*, *Fervidicoccales* and *Thermoplasmatales* have been detected exclusively in

high-temperature oil fields above 50 °C. *Thermococcales* and *Archaeoglobales* are known as thermophilic lineages and have frequently been isolated from oil reservoirs above 70 °C [24, 29, 92]. *Methanobacteriales* (e.g. *Methanothermobacter*), *Thermococcales* (e.g. *Thermococcus*), *Methanococclaesk* and *Archaeoglobales* were most abundant in high-temperature oil fields [9]. *Methanosarcinales*, *Methanomicrobiales* (e.g. *Methanocorpusculum* and *Methanolinea*), *Desulfurococcales*, and *Methanocellales* were mostly isolated from oil reservoirs below 50 °C.

Cai *et. al.* investigated four production wells comprising a range of 1620 m to 2470 m depth and 35.5 °C to 69.0 °C [1]. They found an increasing relative abundance of genes related to the degradation of aromatic compounds (*nahA*, *HBH*, and *pobA*), carbon cycling and metabolism of other organic compounds with decreasing temperature and depth of oil-containing stratum. Stress response (heat shock), antibiotic resistance, and sulfur metabolism associated genes decreased with decreasing temperature [1].

Taken together, a correlation between functional gene occurrence and reservoir temperature has already been detected in nature. Oil degradation is highest at lower temperatures and reservoirs are more or less sterile at temperatures above 80 °C. Temperature is one of the major factors influencing microbial community composition and function in oil reservoirs producing trends on a genus level but not always on an order level. In general, it can be concluded that *Proteobacteria* (*Alpha-*, *Gamma-*, and *Epsilonproteobacteria*) and *Euryarchaeota* are ubiquitous in oil reservoirs across all temperature ranges. Sulfate-reducing bacteria (SRB) thrive from 4 to 85 °C [9]. Hence, no general predictions can be made on phylum or class level based on the oil reservoir temperature. On order and genus level, however, we can see clear temperature preferences as some orders, most of them archaea, were only isolated from high temperature reservoirs. Thus, they may serve as indicators for determination of *in situ* reservoir temperature. However, a general prediction of microbial community composition based on temperature alone is still not possible.

In oil reservoirs, salinity concentrations range from almost fresh- to salt-saturated water. Even though salinity and pH have been much less examined than temperature, they also have a high impact on microbial communities in oil by affecting growth and limiting bacterial activity. It was found that *Clostridia* correlated with low salinity of 3.8%, while *Petrotoga* and *Desulfotomaculum* species were mostly found in samples with a higher salinity of 7.2% [24]. It was suggested that hydrocarbon-degradation by *Desulfotomaculum* species may occur even under relatively high salinity conditions [24]. The amount of microbes isolated from oil fields decreased with increasing reservoir salinity above 10% [10]. Sulfate-reducing bacteria

where found to resist wide ranges of salinity from 0 to 17% [9]. Manipulating the salinity of the injection water during oil-production to NaCl concentrations above 12% inhibits microbial H_2S production [8]. The analysis of two different pits from the Rancho La Brea Tar Pits in Los Angeles indicated that site-specific differences in salinity were highly correlated with microbial community structures within the asphalt [26]. Salt concentrations in oil reservoirs affected methanogenic oil biodegradation as hydrogenotrophic methanogenesis from CO_2 with H_2 was only measured up to a salt concentration of 9%, *in situ* [86, 98] (Table 3).

The *in situ* pH values of oil reservoirs typically range from 3-7 [3]. Sulfate-reducing bacteria where not only found to resist wide ranges of salinity but also a wide range of pH values, from 4-9.5 [9]. A site-specific correlation between pH and microbial community structures was detected for two different pits from the Rancho La Brea Tar Pits in Los Angeles [99]. A study across 22 geographically separated oil reservoirs in China showed that *Alphaproteobacteria*, *Deltaproteobacteria* and *Actinobacteria* were most abundant in neutral to alkaline reservoirs with pH values between 7.0-8.2. *Pseudomonas* correlated with decreasing pH value of formation brine in the range of 5.5-7.6. *Gammaproteobacteria*, *Betaproteobacteria,* and *Epsilonproteobacteria* preferred even more acidic environments and were detected in reservoirs with pH values of 5.5–6.5 [29] (Table 3).

Table 3 Bacteria and Archaea associated to salinity and pH in petroleum reservoirs.

phylum / class	**order / genus**	**prefered salinity and pH**	**reference**
Euryarchaeota / *Methanobacteria*	*Methanobacteriales* / *Methanothermobacter*	acidic pH	[29]
Proteobacteria / *Gammaproteobacteria*	*Pseudomonadales* / *Pseudomonas*	acidic pH (5.5-7.6)	
Proteobacteria / *Betaproteobacteria*		acidic pH (5.5-6.5)	
Proteobacteria / *Epsilonproteobacteria*		acidic pH (5.5-6.5)	
Proteobacteria / *Gammaproteobacteria*		acidic pH	
Euryarchaeota / *Archaeoglobi*	*Archaeoglobales* / *Archaeoglobus*	alkaline pH	
Proteobacteria / *Deltaproteobacteria*	*Desulfuromonadales* / *Desulfuromonas*	alkaline pH	
Euryarchaeota / *Methanococci*	*Methanococcales* / *Methanococcus*	alkaline pH	
Euryarchaeota / *Methanomicrobia*	*Methanomicrobiales* / *Methanocorpusculum*	alkaline pH	

Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanocalculus*	alkaline pH	
Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanoculleus*	alkaline pH	
Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanolinea*	alkaline pH	
Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanosaeta*	alkaline pH	
Euryarchaeota / Methanomicrobia	*Methanosarcinales / Methanolobus*	alkaline pH	
Proteobacteria / Alphaproteobacteria	*Rhodobacterales / Paracoccus*	alkaline pH (7.0-8.2)	
Actinobacteria		alkaline pH (7.0-8.0)	
Proteobacteria / Alphaproteobacteria		alkaline pH (7.0-8.0)	
Firmicutes / Clostridia	*Clostridiales / Desulfotomaculum*	higher salinity	[24]
Proteobacteria / Deltaproteobacteria	*Desulfovibrionales / Desulfovermiculus halophilus*	higher salinity	[94]
Firmicutes / Clostridia	*Halanaerobiales / Haloanaerobium*	higher salinity	[96, 100]
Euryarchaeota / Methanococci	*Methanococcales / Methanothermococcus*	higher salinity	[29]
Thermotogae / Thermotogae	*Petrotogales / Petrotoga*	higher salinity	[24]
Euryarchaeota / Methanobacteria	*Methanobacteriales / Methanobacterium*	lower salinity	[93, 97]
Euryarchaeota / Methanomicrobia	*Methanomicrobiales / Methanoplanus*	lower salinity	[101]

Viruses in oil reservoirs

Viruses are known to have a major impact on microbial communities and their ecology [1, 102-108]. By lysing their bacterial hosts, bacteriophages cause the release and turnover of nutrients such as proteins and nucleic acids [105, 107]. Lysis can also result in changes in the bacterial community composition known as the ’killing the winner’ hypothesis [105, 106, 109], meaning that host-specific predators (viruses) attack a bacterial population if the bacterial density increases over a certain threshold abundance. It thus prevents a species from emerging and maintains the coexistence of all species in the system [110]. Killing the winner models predict that density- and frequency-dependent viral predation suppresses rapidly growing hosts, which leads to increasing host diversity [111]. Nevertheless, phages can also integrate into the host’s genome as prophages (lysogeny). The ‘piggyback the winner’ model predicts relationships between virus-like-particles and host densities. The main advantage for the phage lies in continuous proliferation by the regular host cell growth and division, without

killing the host [111, 112]. Prophages protect the microbial cells from new infections by closely related phages. Due to the protection from lysis and other infections, prophages can drive bacterial evolution by transfer of genetic information between multiple hosts and promote thereby an increased diversity [107, 111-113]. The gene transfer can affect the capacity for biofilm formation, the abilities of hydrocarbon-degradation, antibiotic resistance or the virulence in a positive or a negative way [103, 114].

To date, the natural occurrence of bacteriophages and their interactions with bacteria in natural oil reservoirs has not been studied in much detail. As far as we are aware, no viruses have been directly isolated from an oil reservoir. Only studies at a genomic level have revealed hints of the presence of viruses. Oil-water mixture samples from a production well of the water-flooded Chinese Qinghai oilfield were compared in taxonomic and functional compositions of the microbial communities in the oil and water phases by pyrosequencing and application of a GeoChip4.0 [1]. In that study, 38 of 40 detectable virus genes were found. Three of the detected genes showed significant differences in abundance between crude oil and water phase. Holin type 3 for bacterial lysis was more abundant in the oil phase, while the host recognition T2 type and the sliding clamp T4 for replication were both greater in the water phase. Because of the higher abundance of phage genes in the water phase, it was hypothesized that microbes are protected from phages by the oil phase [1]. Yet, holin type 3 abundance was higher in oil than in water phase, indicating phage-interactions and bacterial lysis directly within the oil phase [1]. As a paradigm of life, microorganisms can only live in a water phase. Thus, microbes in the oil phase are either physically partitioned into the oil during the production process, where they cannot live, or they are present in microdroplets of water dispersed in the oil. In the latter case, they would again be subject to viruses if these are present in the droplet. Another genomic study on *Thermococcus sibiricus* isolated from oil concluded that the oil environment is poorly invaded by bacteriophages because they only found a single CRISPR containing 24 repeat spacer units. However, it is known that other species of the order *Thermococcales* harbor multiple CRISPR loci carrying more repeat spacer units [115].

The studies presented have shown that viruses occur in oil reservoirs. However, questions concerning their ecological importance and the extent to which they shape and control microbial communities and processes remain to be elucidated. Studies on oil-contaminated waters such as spills and plumes and the correlated bioremediation processes propose a phage-driven microbial loop [105, 116]. The authors proposed that phages ensure a persistent nutritional biomass turnover enabling bacterial hydrocarbon degradation [117, 118]. We could

not find any support of either ‘Killing the winner’ or ‘Piggyback-the-winner’ processes in natural oil reservoirs in the literature indicating an open field for ecological research.

4.1.4 Conclusion

Microbial oil degradation in deep subsurface oil reservoirs mainly takes place at the so-called oil-water transition zone (OWTZ) or oil-water interface. Even, if the OWTZ is a degradation hotspot, we propose that microbial oil degradation also takes place in small water-saturated parts of the rock containing actively living microbes or even in the thin water film of water wet reservoirs. Thus, biodegradation is distributed in a gradient through the entire oil field starting from the OWTZ and following the water content to the top of the reservoir. Therefore, the water inclusions should be considered as having a notable impact on overall oil degradation process in the deep subsurface.

Water samples from oil reservoirs obtained by pumping comprise a foamy mixture of oil, formation or injection water, and gas. We propose to examine both the water and the emulsified water within the oil phase of a sample to get a better picture of the entire community present in an oil reservoir. However, production water samples cannot provide information about the real distribution of the microorganisms in the deep biosphere but may provide insight into which organisms are involved in oil degradation.

Another important factor are syntrophic interactions between different microorganisms. Often, planktonic microorganisms do not have access to both electron donor and electron acceptor as prerequisites for microbial energy conservation, especially fermenting bacteria and methanogenic archaea. Therefore, we suggest that the methanogenic degradation of oil mainly takes place in mutualistic microbial consortia organized in biofilms where fermenting microbes transfer electrons either directly or indirectly to the methanogens. In laboratory experiments, many oil-degrading enrichment cultures and isolates build biofilms on the surfaces of alkane phases or PAH´s. Since known advantages of biofilms include protection against toxic compounds and desiccation and syntrophic electron transfer between fermenting organisms and methanogenic archaea, we propose that life in deep subsurface oil reservoirs is arranged predominantly in biofilms.

An important influence regarding degradation rates of crude oil is reservoir temperature, with most inhabited reservoirs ranging from mesophilic to thermophilic conditions. The largest microbial diversity occurs at moderate temperatures of up to 55 °C, where higher metabolic activity and increased abundance of genes involved in carbon cycling and the

degradation of aromatic and other organic compounds occurs. Above ~80 °C, oil reservoirs are considered to be sterile.

Oil reservoir temperature gives us an idea of microbial temperature preferences on a genus level but not always on an order level and so far no general predictions can be made about the phylum or class level. Some organisms may serve as indicators for *in situ* reservoir temperature determination. However, general predictions on microbial community composition based on temperature alone are not feasible.

Viruses could be another important factor in oil reservoirs. Regarding the 'killing the winner' and the 'piggyback-the-winner' hypotheses, viruses could have an impact on shaping microbial communities and function, but no concrete evidence from oil reservoirs has been provided thus far.

Oil reservoirs provide an exceptional habitat for microorganisms, influenced by abiotic and biotic factors. Over the last decades, knowledge on the oil microbiome has grown but the function of the microorganisms described and the principles of the microbial oil degradation process still constitute open questions.

Funding sources

This work was supported by the European Research Council (ERC) [grant number 666952-EcOILogy] and the German Research Foundation (DFG) [grant number BR 5493/1-1].

Conflict of interest

The authors declare no conflict of interest.

4.1.5 REFERENCES

[1] Cai M, Nie Y, Chi CQ, Tang YQ, Li Y, Wang XB, et al. Crude oil as a microbial seed bank with unexpected functional potentials. Sci Rep 2015;5:16057.

[2] Silva TR, Verde LCL, Neto EVS, Oliveira VM. Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. Int Biodeter Biodegrad 2013;81:57–70.

[3] Magot M, Ollivier B, Patel BK. Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 2000;77:103–16.

[4] Turhan I, Hacihasanoglu E, Soytas U. Oil prices and emerging market exchange rates. Emerg Mark Financ Tr 2013;49:21–36.

[5] Head IM, Jones DM, Larter SR. Biological activity in the deep subsurface and the origin of heavy oil. Nature 2003;426:344–52.

[6] Liebensteiner MG, Tsesmetzis N, Stams AJ, Lomans BP. Microbial redox processes in deep subsurface environments and the potential application of (per)chlorate in oil reservoirs. Front Microbiol 2014;5:428.

[7] Timmis KN, McGenity T, Van Der Meer JR, de Lorenzo V. Handbook of hydrocarbon and lipid microbiology. Berlin: Springer; 2010.

[8] Youssef N, Elshahed MS, McInerney MJ. Microbial processes in oil fields: culprits, problems, and opportunities. Adv Appl Microbiol 2009;66:141–251.

[9] Li XX, Mbadinga SM, Liu JF, Zhou L, Yang SZ, Gu JD, et al. Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. Int Biodeter Biodegrad 2017;120:170–85.

[10] Roling WF, Head IM, Larter SR. The microbiology of hydrocarbon degradation in subsurface petroleum reservoirs: perspectives and prospects. Res Microbiol 2003;154:321–8.

[11] Larter S, Wilhelms A, Head I, Koopmans M, Aplin A, Di Primio R, et al. The controls on the composition of biodegraded oils in the deep subsurface—part 1: biodegradation rates in petroleum reservoirs. Org Geochem 2003;34:601–13.

[12] Kobayashi H, Endo K, Sakata S, Mayumi D, Kawaguchi H, Ikarashi M, et al. 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 2012;113:204–10.

[13] Ridley CM, Voordouw G. Aerobic microbial taxa dominate deep subsurface cores from the Alberta oil sands. FEMS Microbiol Ecol 2018;94. fiy073.

[14] Sierra-Garcia IN, de Oliveira VM. Microbial hydrocarbon degradation: efforts to understand biodegradation in petroleum reservoirs. Biodegradation-engineering and Technology. InTech; 2013.

[15] Abbasnezhad H, Gray M, Foght JM. Influence of adhesion on aerobic biodegradation and bioremediation of liquid hydrocarbons. Appl Microbiol Biotechnol 2011;92:653–75.

[16] Nazina T, Ivanova A, Golubeva O, Ibatullin R, Belyaev S, Ivanov M. Occurrence of sulfate-and iron-reducing bacteria in stratal waters of the Romashkinskoe oil field. Microbiology-New York 1995;64:203–8.

[17] Nazina T, Shestakova N, Pavlova N, Tatarkin Y, Ivoilov V, Khisametdinov M, et al. Functional and phylogenetic microbial diversity in formation waters of a lowtemperature carbonate petroleum reservoir. Int Biodeterior Biodegrad 2013;81:71–81.

[18] Semple K, Westlake D. Characterization of iron-reducing Alteromonas putrefaciens strains from oil field fluids. Can J Microbiol 1987;33:366–71.

[19] Greene AC, Patel BK, Sheehy AJ. Deferribacter thermophilus gen. nov., sp. nov., a novel thermophilic manganese-and iron-reducing bacterium isolated from a petroleum reservoir. Int J Syst Bacteriol 1997;47:505–9.

[20] Feng W-W, Liu J-F, Gu J-D, Mu B-Z. Nitrate-reducing community in production water of three oil reservoirs and their responses to different carbon sources revealed by nitrate-reductase encoding gene (napA). Int Biodeter Biodegrad 2011;65:1081–6.

[21] Lovley DR, Chapelle FH. Deep subsurface microbial processes. Rev Geophys 1995;33:365–81.

[22] Zengler K, Richnow HH, Rossello-Mora R, Michaelis W, Widdel F. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 1999;401:266–9.

[23] Chilingar GV, Yen TF. Bitumens, asphalts, and tar sands. Developments in petroleum science xii. New York: Elsevier Scientific Pub Co; 1978. p. 331.

[24] Vigneron A, Alsop EB, Lomans BP, Kyrpides NC, Head IM, Tsesmetzis N. Succession in the petroleum reservoir microbiome through an oil field production lifecycle. ISME J 2017;11:2141–54.

[25] Korenblum E, Souza DB, Penna M, Seldin L. Molecular analysis of the bacterial communities in crude oil samples from two brazilian offshore petroleum platforms. Int J Microbiol 2012;2012:156537.

[26] Kim JS, Crowley DE. Microbial diversity in natural asphalts of the rancho La brea tar pits. Appl Environ Microbiol 2007;73:4579–91.

[27] Meckenstock RU, von Netzer F, Stumpp C, Lueders T, Himmelberg AM, Hertkorn N, et al. Oil biodegradation. Water droplets in oil are microhabitats for microbial life. Science 2014;345:673–6.

[28] Wang LY, Ke WJ, Sun XB, Liu JF, Gu JD, Mu BZ. Comparison of bacterial community in aqueous and oil phases of water-flooded petroleum reservoirs using pyrosequencing and clone library approaches. Appl Microbiol Biotechnol 2014;98:4209–21.

[29] Gao P, Tian H, Wang Y, Li Y, Li Y, Xie J, et al. Spatial isolation and environmental factors drive distinct bacterial and archaeal communities in different types of petroleum reservoirs in China. Sci Rep 2016;6:20174.

[30] Tang YQ, Li Y, Zhao JY, Chi CQ, Huang LX, Dong HP, et al. Microbial communities in long-term, water-flooded petroleum reservoirs with different *in situ* temperatures in the Huabei Oilfield, China. PLoS One 2012;7:e33535.

[31] Wang LY, Duan RY, Liu JF, Yang SZ, Gu JD, Mu BZ. Molecular analysis of the microbial community structures in water-flooding petroleum reservoirs with different temperatures. Biogeosciences 2012;9:4645–59.

[32] Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annu Rev Microbiol 2002;56:187–209.

[33] Obuekwe CO, Al-Zarban SS. Bioremediation of crude oil pollution in the Kuwaiti desert: the role of adherent microorganisms. Environ Int 1998;24:823–34.

[34] Raghukumar C, Vipparty V, David JJ, Chandramohan D. Degradation of crude oil by marine cyanobacteria. Appl Microbiol Biotechnol 2001;57:433–6.

[35] Schneiker S, Martins dos Santos VA, Bartels D, Bekel T, Brecht M, Buhrmester J, et al. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium Alcanivorax borkumensis. Nat Biotechnol 2006;24:997–1004.

[36] Vert M, Doi Y, Hellwich KH, Hess M, Hodge P, Kubisa P, et al. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure Appl Chem 2012(84):377–408.

[37] Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623–33.

[38] Frølund B, Palmgren R, Keiding K, Nielsen PH. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res 1996;30:1749–58.

[39] Morgan JW, Forster CF, Evison L. A comparative-study of the nature of biopolymers extracted from anaerobic and activated sludges. Water Res 1990;24:743–50.

[40] Persat A, Nadell CD, Kim MK, Ingremeau F, Siryaporn A, Drescher K, et al. The mechanical world of bacteria. Cell 2015;161:988–97.

[41] Wingender J, Neu TR, Flemming H-C. What are bacterial extracellular polymeric substances? Microbial extracellular polymeric substances. Springer; 1999. p. 1–19.

[42] Barkay T, Navon-Venezia S, Ron EZ, Rosenberg E. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiol 1999;65:2697–702.

[43] Calvo C, Martinez-Checa F, Toledo FL, Porcel J, Quesada E. Characteristics of bioemulsifiers synthesised in crude oil media by Halomonas eurihalina and their effectiveness in the isolation of bacteria able to grow in the presence of hydrocarbons. Appl Microbiol Biotehnol 2002;60:347–51.

[44] Eriksson M, Dalhammar G, Mohn WW. Bacterial growth and biofilm production on pyrene. FEMS Microbiol Ecol 2002;40:21–7.

[45] Golyshin PN, Chernikova TN, Abraham WR, Lunsdorf H, Timmis KN, Yakimov MM. Oleiphilaceae fam. nov., to include Oleiphilus messinensis gen. nov., sp nov., a novel marine bacterium that obligately utilizes hydrocarbons. Int J Syst Evol Microbiol 2002;52:901–11.

[46] Johnsen AR, Karlson U. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. Appl Microbiol Biotechnol 2004;63:452–9.

[47] Klein B, Bouriat P, Goulas P, Grimaud R. Behavior of Marinobacter hydrocarbonoclasticus SP17 cells during initiation of biofilm formation at the alkane–water interface. Biotechnol Bioeng 2010;105:461–8.

[48] Navon-Venezia S, Zosim Z, Gottlieb A, Legmann R, Carmeli S, Ron EZ, et al. Alasan, a new bioemulsifier from Acinetobacter radioresistens. Appl Environ Microbiol 1995;61:3240–4.

[49] Billings N, Birjiniuk A, Samad TS, Doyle PS, Ribbeck K. Material properties of biofilms—a review of methods for understanding permeability and mechanics. Rep Prog Phys 2015;78:036601.

[50] Flemming HC, Wingender J. Biofilme—die bevorzugte Lebensform der Bakterien: Flocken, Filme und Schlämme. Biol Unserer Zeit 2001;31:169–80.

[51] Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 2016;14:563–75.

[52] Mah TF. Biofilm-specific antibiotic resistance. Future Microbiol 2012;7:1061–72.

[53] Klein B, Grossi V, Bouriat P, Goulas P, Grimaud R. Cytoplasmic wax ester accumulation during biofilm-driven substrate assimilation at the alkane–water interface by Marinobacter hydrocarbonoclasticus SP17. Res Microbiol 2008;159:137–44.

[54] Jimenez N, Richnow HH, Vogt C, Treude T, Kruger M. Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. J Mol Microbiol Biotechnol 2016;26:227–42.

[55] Varjani SJ, Gnansounou E. Microbial dynamics in petroleum oilfields and their relationship with physiological properties of petroleum oil reservoirs. Bioresource Technol 2017;245:1258–65.

[56] Berdugo-Clavijo C, Gieg LM. Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production waters. Front Microbiol 2014;5:197.

[57] Gieg LM, Fowler SJ, Berdugo-Clavijo C. Syntrophic biodegradation of hydrocarbon contaminants. Curr Opin Biotechnol 2014;27:21–9.

[58] Hu P, Tom L, Singh A, Thomas BC, Baker BJ, Piceno YM, et al. Genome-resolved metagenomic analysis reveals roles for candidate Phyla and other microbial community members in biogeochemical transformations in oil reservoirs. MBio 2016;7:e01669–15.

[59] Liu YF, Galzerani DD, Mbadinga SM, Zaramela LS, Gu JD, Mu BZ, et al. Metabolic capability and *in situ* activity of microorganisms in an oil reservoir. Microbiome 2018;6:5.

[60] Meyer B, Kuehl J, Deutschbauer AM, Price MN, Arkin AP, Stahl DA. Variation among Desulfovibrio species in electron transfer systems used for syntrophic growth. J Bacteriol 2013;195:990–1004.

[61] Plugge CM, Zhang W, Scholten JC, Stams AJ. Metabolic flexibility of sulfate-reducing bacteria. Front Microbiol 2011;2:81.

[62] Gieg LM, Davidova IA, Duncan KE, Suflita JM. Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. Environ Microbiol 2010;12:3074–86.

[63] Gray ND, Sherry A, Grant RJ, Rowan AK, Hubert CR, Callbeck CM, et al. The quantitative significance of Syntrophaceae and syntrophic partnerships in methanogenic degradation of crude oil alkanes. Environ Microbiol 2011;13:2957–75.

[64] Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Aitken CM, et al. Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 2008;451:176–80.

[65] Schink B. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 1997;61:262–80.

[66] Sieber JR, Le HM, McInerney MJ. The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. Environ Microbiol 2014;16:177–88.

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

[68] Toth CRA, Gieg LM. Time course-dependent methanogenic crude oil biodegradation: dynamics of fumarate addition metabolites, biodegradative genes, and microbial community composition. Front Microbiol 2017;8:2610.

[69] McInerney MJ, Sieber JR, Gunsalus RP. Syntrophy in anaerobic global carbon cycles. Curr Opin Biotechnol 2009;20:623–32.

[70] Mesle M, Dromart G, Oger P. Microbial methanogenesis in subsurface oil and coal. Res Microbiol 2013;164:959–72.

[71] Whiticar MJ, Faber E, Schoell M. Biogenic methane formation in marine and freshwater environments - CO_2 reduction vs acetate fermentation isotope evidence. Geochim Cosmochim Ac 1986;50:693–709.

[72] Piceno YM, Reid FC, Tom LM, Conrad ME, Bill M, Hubbard CG, et al. Temperature and injection water source influence microbial community structure in four Alaskan North Slope hydrocarbon reservoirs. Front Microbiol 2014;5:409.

[73] Fowler SJ, Toth CR, Gieg LM. Community structure in methanogenic enrichments provides insight into syntrophic interactions in hydrocarbon-impacted environments. Front Microbiol 2016;7:562.

[74] Sierra-Garcia IN, Dellagnezze BM, Santos VP, Chaves BM, Capilla R, Santos Neto EV, et al. Microbial diversity in degraded and non-degraded petroleum samples and comparison across oil reservoirs at local and global scales. Extremophiles 2017;21:211–29.

[75] Wang LY, Gao CX, Mbadinga SM, Zhou L, Liu JF, Gu JD, et al. Characterization of an alkane-degrading methanogenic enrichment culture from production water of an oil reservoir after 274 days of incubation. Int Biodeter Biodegradation 2011;65:444–50.

[76] Qin QS, Feng DS, Liu PF, He Q, Li X, Liu AM, et al. Metagenomic characterization of candidatus Smithella cisternae strain M82_1, a syntrophic alkane-degrading Bacteria, Enriched from the Shengli Oil Field. Microbes Environ 2017;32:234–43.

[77] Rotaru AE, Shrestha PM, Liu F, Markovaite B, Chen S, Nevin KP, et al. Direct interspecies electron transfer between Geobacter metallireducens and Methanosarcina barkeri. Appl Environ Microbiol 2014;80:4599–605.

[78] Rotaru AE, Shrestha PM, Liu FH, Shrestha M, Shrestha D, Embree M, et al. A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. Energy Environ Sci 2014;7:408–15.

[79] Kouzuma A, Kato S, Watanabe K. Microbial interspecies interactions: recent findings in syntrophic consortia. Front Microbiol 2015;6:477.

[80] Kaster KM, Bonaunet K, Berland H, Kjeilen-Eilertsen G, Brakstad OG. Characterisation of culture-independent and -dependent microbial communities in a high-temperature offshore chalk petroleum reservoir. Antonie Van Leeuwenhoek 2009;96:423–39.

[81] Lenchi N, Inceoglu O, Kebbouche-Gana S, Gana ML, Lliros M, Servais P, et al. Diversity of microbial communities in production and injection waters of Algerian oilfields revealed by 16S rRNA gene amplicon 454 pyrosequencing. PLoS One 2013;8:e66588.

[82] Kowalewski E, Rueslåtten I, Steen K, Bødtker G, Torsæter O. Microbial improved oil recovery—bacterial induced wettability and interfacial tension effects on oil production. J Petrol Sci Eng 2006;52:275–86.

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

[84] Fedorak PM, Westlake DW. Microbial degradation of alkyl carbazoles in Norman wells crude oil. Appl Environ Microbiol 1984;47:858–62.

[85] Huang HP, Bowler BFJ, Zhang ZW, Oldenburg TBP, Larter SR. Influence of biodegradation on carbazole and benzocarbazole distributions in oil columns from the Liaohe basin, NE China. Org Geochem 2003;34:951–69.

[86] Head IM, Gray ND, Larter SR. Life in the slow lane; biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front Microbiol 2014;5:566.

[87] Wang LY, Ke WJ, Sun XB, Liu JF, Gu JD, Mu BZ. Comparison of bacterial community in aqueous and oil phases of water-flooded petroleum reservoirs using pyrosequencing and clone library approaches. Appl Microbiol Biotechnol 2014;98:4209–21.

[88] Bernard FP, Connan J, Magot M. Indigenous microorganisms in connate water of many oil fields: a new tool in exploration and production techniques. SPE annual technical conference and exhibition. Washington, D.C: Society of Petroleum Engineers; 2013.

[89] Stetter KO. Extremophiles and their adaptation to hot environments. FEBS Lett 1999;452:22–5.

[90] Mayumi D, Mochimaru H, Yoshioka H, Sakata S, Maeda H, Miyagawa Y, et al. Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). Environ Microbiol 2011;13:1995–2006.

[91] Lin JZ, Hao B, Cao GZ, Wang J, Feng Y, Tan XM, et al. A study on the microbial community structure in oil reservoirs developed by water flooding. J Petrol Sci Eng 2014;122:354–9.

[92] Gittel A, Sorensen KB, Skovhus TL, Ingvorsen K, Schramm A. Prokaryotic community structure and sulfate reducer activity in water from high-temperature oil reservoirs with and without nitrate treatment. Appl Environ Microbiol 2009;75:7086–96.

[93] Belyaev SS, Obraztsova AY, Laurinavichus KS, Bezrukova LV. Characteristics of rod-shaped methane-producing bacteria from an oil pool and description of methanobacterium-ivanovii sp-nov. Microbiology 1986;55:821–6.

[94] Belyakova EV, Rozanova EP, Borzenkov IA, Tourova TP, Pusheva MA, Lysenko AM, et al. The new facultatively chemolithoautotrophic, moderately halophilic, sulfate-reducing bacterium Desulfovermiculus halophilus gen. nov., sp nov., isolated from an oil field. Microbiology 2006;75:161–71.

[95] Obraztsova AY, Shipin OV, Bezrukova LV, Belyaev SS. Properties of the coccoid methylotrophic methanogen, methanococcoides-euhalobius sp-nov. Microbiology 1987;56:523–7.

[96] Bhupathiraju VK, McInerney MJ, Woese CR, Tanner RS. Haloanaerobium kushneri sp. nov., an obligately halophilic, anaerobic bacterium from an oil brine. Int J Syst Bacteriol 1999;3(49 Pt):953–60.

[97] Davydovacharakhchyan IA, Kuznetsova VG, Mityushina LL, Belyaev SS. Methaneforming Bacilli from oil-fields of Tataria and Western Siberia. Microbiology 1992;61:202–7.

[98] Gray ND, Sherry A, Larter SR, Erdmann M, Leyris J, Liengen T, et al. Biogenic methane production in formation waters from a large gas field in the North Sea. Extremophiles 2009;13:511–9.

[99] Kim JS, Crowley DE. Microbial diversity in natural asphalts of the rancho la brea tar pits. Appl Environ Microbiol 2007;73:4579–91.

[100] Ravot G, Magot M, Ollivier B, Patel BK, Ageron E, Grimont PA, et al. Haloanaerobium congolense sp. nov., an anaerobic, moderately halophilic, thiosulfate- and sulfur-reducing bacterium from an African oil field. FEMS Microbiol Lett 1997;147:81–8.

[101] Ollivier B, Cayol JL, Patel BK, Magot M, Fardeau ML, Garcia JL. Methanoplanus petrolearius sp. nov., a novel methanogenic bacterium from an oil-producing well. FEMS Microbiol Lett 1997;147:51–6.

[102] Brockhurst MA, Fenton A, Roulston B, Rainey PB. The impact of phages on interspecific competition in experimental populations of bacteria. BMC Ecol 2006;6:19.

[103] De Smet J, Hendrix H, Blasdel BG, Danis-Wlodarczyk K, Lavigne R. Pseudomonas predators: understanding and exploiting phage-host interactions. Nat Rev Microbiol 2017;15:517–30.

[104] Koskella B, Brockhurst MA. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol Rev 2014;38:916–31.

[105] Rosenberg E, Bittan-Banin G, Sharon G, Shon A, Hershko G, Levy I, et al. The phage-driven microbial loop in petroleum bioremediation. Microb Biotechnol 2010;3:467–72.

[106] Shapiro OH, Kushmaro A, Brenner A. Bacteriophage predation regulates microbial abundance and diversity in a full-scale bioreactor treating industrial wastewater. ISME J 2010;4:327–36.

[107] Weinbauer MG, Rassoulzadegan F. Are viruses driving microbial diversification and diversity? Environ Microbiol 2004;6:1–11.

[108] Weitz JS, Poisot T, Meyer JR, Flores CO, Valverde S, Sullivan MB, et al. Phage–bacteria infection networks. Trends Microbiol 2013;21:82–91.

[109] Thingstad TF, Lignell R. Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquat Microb Ecol 1997;13:19–27.

[110] Xue C, Goldenfeld N. Coevolution maintains diversity in the stochastic "Kill the winner" model. Phys Rev Lett 2017;119:268101.

[111] Knowles B, Silveira CB, Bailey BA, Barott K, Cantu VA, Cobian-Guemes AG, et al. Lytic to temperate switching of viral communities. Nature 2016;531:466–70.

[112] Silveira CB, Rohwer FL. Piggyback-the-Winner in host-associated microbial communities. NPJ Biofilms Microbiomes 2016;2:16010.

[113] Tu Q, Yu H, He Z, Deng Y, Wu L, Van Nostrand JD, et al. GeoChip 4: a functional gene-array-based high-throughput environmental technology for microbial community analysis. Mol Ecol Resour 2014;14:914–28.

[114] Herrick JB, StuartKeil KG, Ghiorse WC, Madsen EL. Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. Appl Environ Microb 1997;63:2330–7.

[115] Mardanov AV, Ravin NV, Svetlitchnyi VA, Beletsky AV, Miroshnichenko ML, Bonch-Osmolovskaya EA, et al. Metabolic versatility and indigenous origin of the archaeon Thermococcus sibiricus, isolated from a siberian oil reservoir, as revealed by genome analysis. Appl Environ Microbiol 2009;75:4580–8.

[116] Head IM, Jones DM, Roling WF. Marine microorganisms make a meal of oil. Nat Rev Microbiol 2006;4:173–82.

[117] Lu Z, Deng Y, Van Nostrand JD, He Z, Voordeckers J, Zhou A, et al. Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. ISME J 2012;6:451–60.

[118] McGenity TJ, Folwell BD, McKew BA, Sanni GO. Marine crude-oil biodegradation: a central role for interspecies interactions. Aquat Biosyst 2012;8:10.

4.2 Densely Populated Water Droplets in Heavy-Oil Seeps

M. Pannekens,[a] L. Voskuhl,[a] A. Meier,[a] H. Müller,[a] S. Haque,[b] J. Frösler,[a*] V. S. Brauer,[a] R. U. Meckenstock[a]

[a]Environmental Microbiology and Biotechnology, University of Duisburg-Essen, Essen, Germany

[b]Department of Physics, Faculty of Science and Technology, The University of The West Indies, St. Augustine, Trinidad and Tobago

Citation Pannekens M, Voskuhl L, Meier A, Müller H, Haque S, Frösler J, Brauer VS, Meckenstock RU. 2020. Densely populated water droplets in heavy-oil seeps. Appl Environ Microbiol 86:e00164-20. https://doi.org/10 .1128/AEM.00164-20.

Editor Shuang-Jiang Liu, Chinese Academy of Sciences

Address correspondence to R. U. Meckenstock, rainer.meckenstock@uni-due.de.

* Present address: J. Frösler, IWW Water Center, Applied Microbiology, Mülheim an der Ruhr, Germany.

Received 22 January 2020

Accepted 19 March 2020

Accepted manuscript posted online 27 March 2020

Published 19 May 2020

ABSTRACT 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 that there is microbial life enclosed in microliter-sized water droplets dispersed in heavy oil of Pitch Lake in Trinidad and 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 whether 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, Pitch Lake (Trinidad and Tobago), the La Brea Tar Pits (California, USA), and an oil seep on the McKittrick oil field (California, USA). All three tested oil seeps contained dispersed water droplets. Larger droplets between 1 and 10 µl revealed high cell densities of up to 10^9 cells ml^{-1}. Testing for 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 those of 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 microhabitats in heavy oil and possibly a generic trait of degraded-oil reservoirs.

IMPORTANCE Our results confirmed that small water droplets in oil are densely populated microhabitats containing active microbial communities. Since these microhabitats occurred in three tested oil seeps which are located thousands of kilometers away from each other, such populated water droplets might be a generic trait of biodegraded oil reservoirs and might be involved in the overall oil degradation process. Microbial degradation might thus also take place in water pockets in the oil-bearing oil legs of the reservoir rock rather than only at the oil-water transition zone.

KEYWORDS 16S rRNA sequencing, active degradation, bitumen, core community, life in oil, microhabitat, oil degradation, oil reservoir

4.2.1 INTRODUCTION

The world's oil reservoirs are dominated by heavy oil (hereinafter referred to as 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 like 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, Pitch Lake, located on the island of Trinidad, Trinidad and Tobago. In this natural oil seep, Meckenstock et al. discovered complex microbial communities inhabiting tiny water droplets, 1 to 3 µl in volume, suspended in the oil phase, hereinafter termed 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 droplets, containing indigenous microbiota, ascended directly 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 like *Bacteroidales*, *Rhodospirillales*, and *Sphingomonadales*, as well as methanogenic archaea, indicating hydrogenotrophic methanogenesis as the terminal electron-accepting process (15, 16). The microbial activity in the water droplets indicated that biodegradation in oil reservoirs is not restricted only 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 greater overall oil degradation. These water droplets provide a unique opportunity to get insights into the microbial life and degradation processes in the deep subsurface of oil reservoirs.

Nevertheless, it remained unclear whether such microbial communities entrapped in water droplets are a generic feature of oil reservoirs or only a single observation from 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 were living planktonically 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.

4.2.2 RESULTS

Distribution and density of microorganisms in water droplets. In order to determine the localization of microorganisms in water droplets enclosed in oil from 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 specimens clearly showed microorganisms in these droplets, but cells were only found in water inclusions larger than 10 to 20 µm in diameter (Fig. 1).

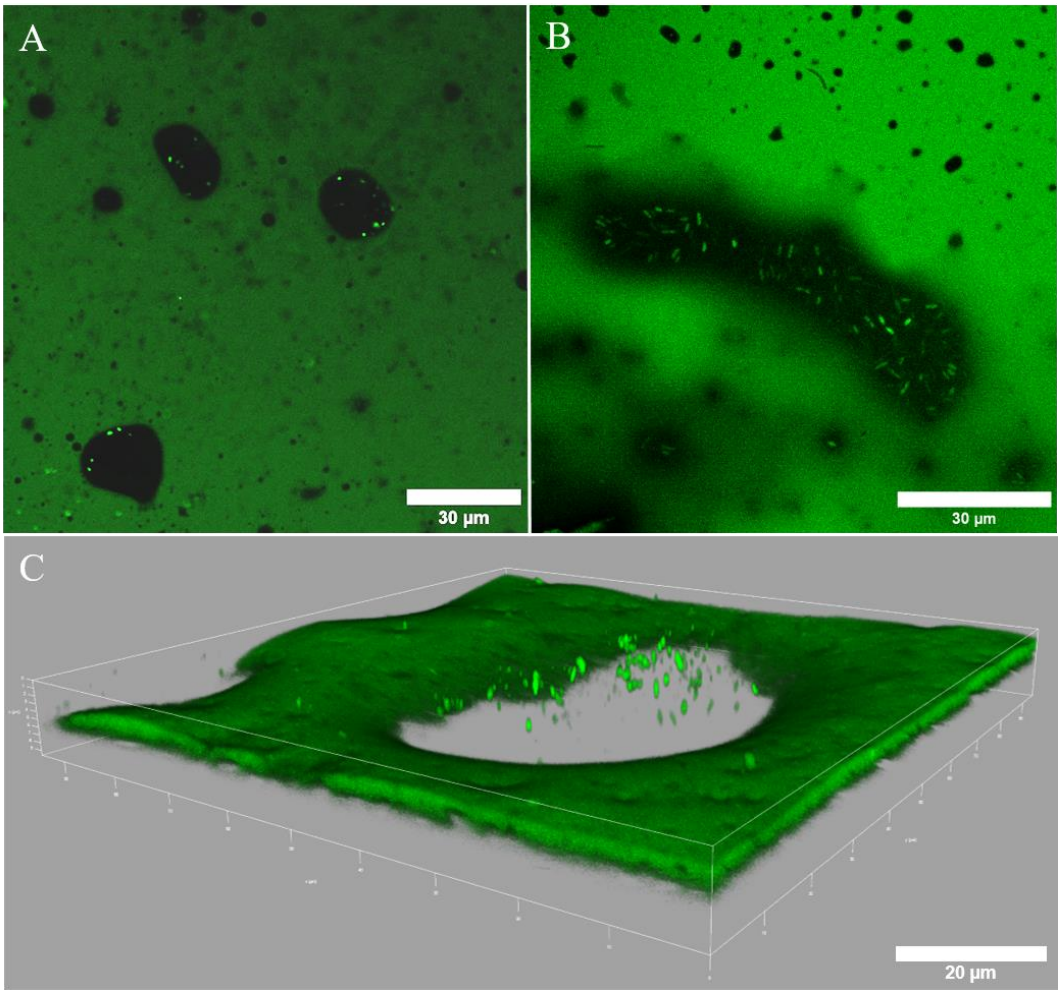


FIG 1 CLSM fluorescence images of natural water droplets (black) dispersed in oil (green) from McKittrick (A, C) and La Brea (B) oil samples. Bright green dots represent microbial

cells stained with Syto 9. (A, B) Two-dimensional view of different water droplets. (C) Three-dimensional view of different water droplets.

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 5.6×10^3 to 1.2×10^6 cells μl^{-1} (Fig. 2). The average cell numbers ranged from 2.6×10^4 cells μl^{-1} (n = 10) in the McKittrick water droplets, over 1.2×10^5 cells μl^{-1} (n = 10) in the ones from Pitch Lake, to 4.5×10^5 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 1.2×10^6 cells μl^{-1}.

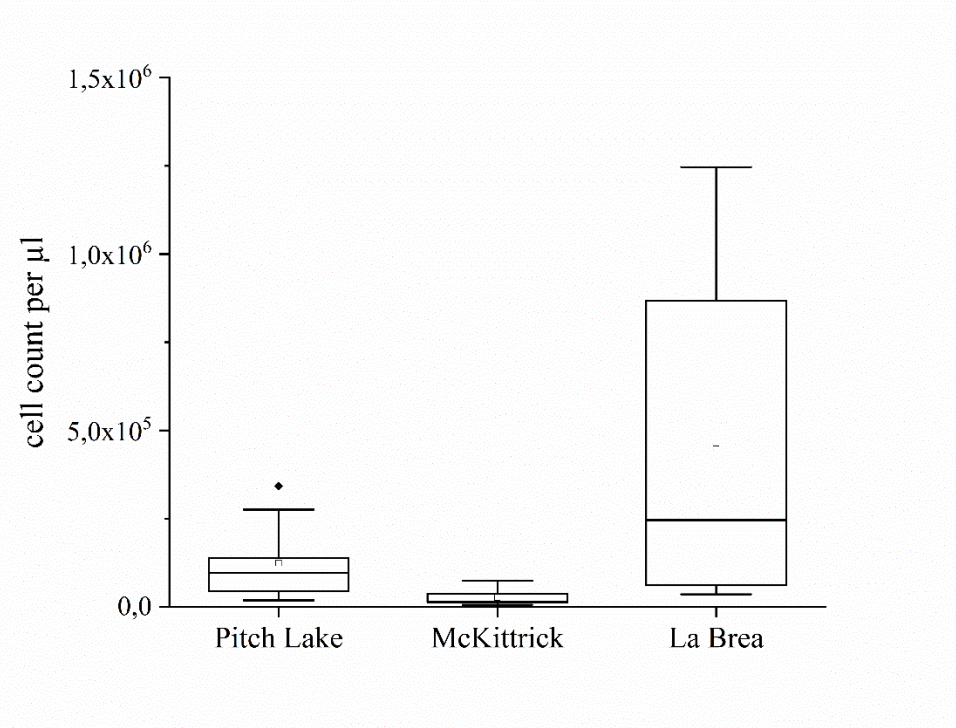


FIG 2 Box plots of total cell counts of isolated water droplets from the three different oil seeps: Pitch Lake (Trinidad and Tobago), McKittrick (CA, USA), and La Brea Tar Pits (CA, USA). In total, 30 droplets (10 of each oil seep) were counted with a Thoma chamber.

According to the fluorescence microscopy results mentioned 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 cocci, diplococci, and filamentous microorganisms were also observed. The epifluorescence counting of filtered droplet water (data not shown) confirmed the counting via Thoma chamber.

Water droplets contain living cells. To analyze whether the observed cells were living microorganisms, we applied LIVE/DEAD staining to differentiate between membrane-intact and membrane-damaged cells (Fig. 3).

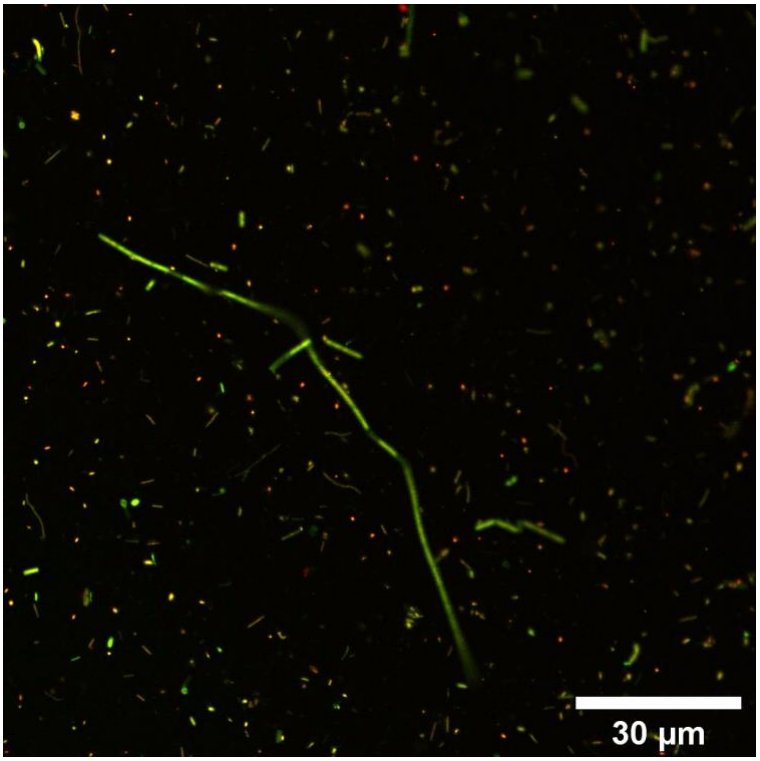


FIG 3 CLSM fluorescence micrograph of a water droplet isolated from the La Brea Tar Pits. The cells were stained with Syto 9 and propidium iodide. Membrane-intact cells appear green, whereas membrane-damaged cells are stained red.

Membrane-intact cells (Fig. 3, green fluorescent signal) were found in all populated droplets. The ratio between membrane-intact and membrane-damaged (Fig. 3, red fluorescent signal) cells varied between water droplets from the three oil seeps. Nevertheless, the average amount of intact cells was around 53% in all three seeps, indicating that substantial amounts 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).

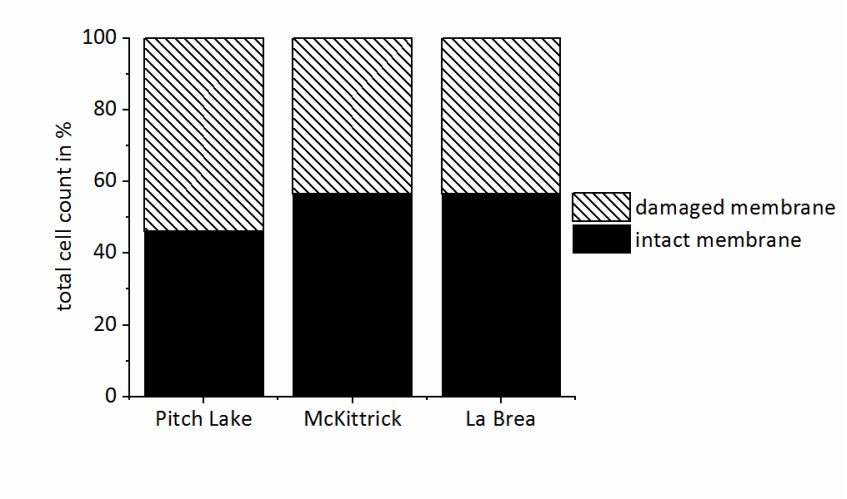


FIG 4 Distribution of membrane-intact and membrane-damaged cells in water droplets of the three tested oil seeps. In total, 197 cells were evaluated from Pitch Lake droplets, 1,394 from McKittrick droplets, and 1,564 from La Brea droplets.

Furthermore, we determined the concentrations of ATP, which is an indicator for active and live cells, in single droplets. Control ATP standards dissolved in water or water extracted from Pitch Lake oil did not indicate either inhibition or enhancement of the signal obtained (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 Pitch Lake, with 21.8 pM, followed by McKittrick, with 194.8 pM, and La Brea, with 492.2 pM.

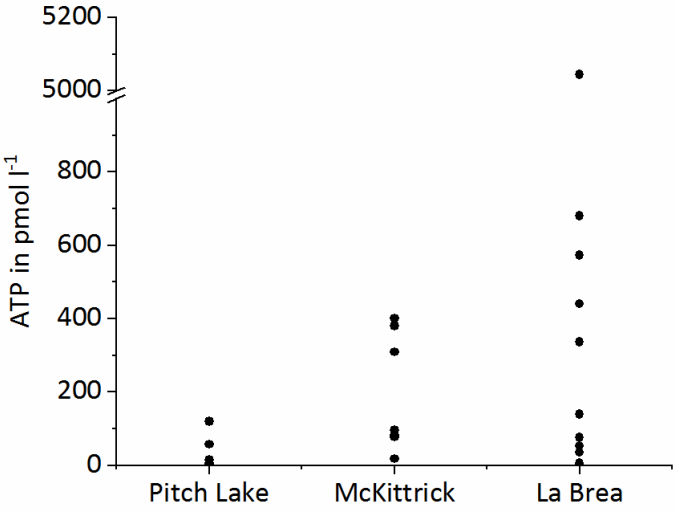


FIG 5 Measured ATP contents in water droplets extracted from the three oil seeps.

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 to 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 operational taxonomic units (OTUs) at a 97% sequence similarity cutoff. Among those, 525 OTUs belonged to 26 bacterial phyla and 33 OTUs to 4 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. The most prominent representatives belonged to the bacterial phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Synergistetes*, *Deferribac teres*, *Thermotogae*, *Chloroflexi*, *Bacteroidia*, and candidate phylum "Atribacteria," while *Euryarchaeota* and *Woesearchaeota* represented the dominant archaeal phyla. OTUs that could not be classified by the RDP classifier were reclassified using BLAST against the nonredundant NCBI nucleotide database (17). Most unclassified OTUs could be assigned to the candidate phyla "Atribacteria" and "Parcubacteria."

The 10 most abundant OTUs in the respective oil seeps 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 extensively by individual OTUs (Table 1). This is supported by the Simpson diversity indices of $D = 0.75 \pm 0.11$ (mean ± standard deviation) 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 droplets, and $H = 2.7 \pm 1.0$ for La Brea droplets indicate the most diverse community in McKittrick droplets.

TABLE 1 The 10 most abundant OTUs within each oil seep, together with their relative abundances in descending order

				Relative abundance of OTUs per oil seep[b]		
OTU	**Family**	**Genus**	**Prevalence ≥ 97%[a]**	**La Brea**	**McKittrick**	**Pitch Lake**
1	*Hydrogenophilaceae*	*Tepidiphilus*		BD	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)	BD
4	*Methanotrichaceae*	*Methanothrix*		1.8 (± 0.13)	BD	BD
5	*Pseudomonadaceae*	*Pseudomonas*		0.93 (± 0.24)	0.03 (± 0.01)	BD
6	*Clostridiales_Incertae_Sedis_XI*	*Soehngenia*	97 %	0.01 (± 0.001)	0.05 (± 0.003)	0.86 (± 0.06)
7	Betaproteobacteria (unclassified)	unclassified		BD	0.7 (± 0.05)	BD
8	*Desulfobulbaceae*	*Desulfoprunum*		0.01 (± 0.002)	0.55 (± 0.05)	BD
9	*Syntrophobacteraceae*	unclassified		BD	0.51 (± 0.08)	BD
10	Woesearchaeota (unclassified)	unclassified		BD	BD	0.56 (± 0.06)
11	*Syntrophorhabdus*	unclassified		0.38 (± 0.06)	BD	BD
12	*Comamonadaceae*	unclassified		BD	0.01 (± 0.002)	0.53 (± 0.17)
13	*Hydrogenophilaceae*	*Tepidiphilus*		0.43 (± 0.04)	0.01 (± 0.002)	BD
14	*Hydrogenophilaceae*	*Thiobacillus*		BD	0.4 (± 0.03)	BD
15	*Deferribacteraceae*	unclassified	97 %	0.02 (± 0.003)	0.08 (± 0.01)	0.55 (± 0.11)
16	*Synergistaceae*	*Anaerobaculum*		0.32 (± 0.03)	0.01 (± 0.003)	BD
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)	BD	BD
19	*Synergistaceae*	*Thermovirga*		BD	BD	0.43 (± 0.02)
20	*Deferribacteraceae*	*Calditerrivibrio*		0.32 (± 0.05)	0.01 (± 0.001)	BD
22	Bacteria (unclassified)	unclassified		0.33 (± 0.03)	0.01 (± 0.001)	BD
23	*Petrotogaceae*	unclassified	97 %	0.01 (± 0.002)	0.003 (± 0.001)	0.39 (± 0.02)
24	*Syntrophaceae*	*Desulfomonile*		BD	0.29 (± 0.04)	BD
25	*Bacillaceae_1*	unclassified	100 %	0.07 (± 0.01)	0.19 (± 0.02)	0.003 (± 0.0003)
26	*Porphyromonadaceae*	*Proteiniphilum*		BD	0.26 (± 0.02)	BD
27	*Bacteroidaceae*	*Bacteroides*		BD	0.23 (± 0.02)	BD
45	*Petrotogaceae*	unclassified		BD	BD	0.15 (± 0.01)
21	*Synergistaceae*	*Anaerobaculum*	100 %	0.17 (± 0.03)	0.05 (± 0.01)	0.1 (± 0.01)
36	Bacteria (unclassified)	unclassified	100 %	0.05 (± 0.01)	0.06 (± 0.01)	0.06 (± 0.003)
52	*Bacillaceae_1*	unclassified	97 %	0.03 (± 0.004)	0.07 (± 0.01)	0.001 (± 0.0002)
38	*Anaerolineaceae*	unclassified	97 %	0.06 (± 0.01)	0.02 (± 0.002)	0.1 (± 0.01)

[a]A core community of 10 OTUs was defined as present in 97 to 100% of the droplets of all three sites (prevalence). As part of the core community, OTUs 21, 36, 52, and 38 were added to the table regardless of their respective relative abundance.

[b]The 10 most abundant OTUs within each oil seep are marked in light gray. OTUs which were not detected in the particular seep or were detected with an abundance of <0.009% are marked as below detection (BD). Data depict the mean value and standard deviation of the relative abundances of the respective organism in all droplets from one site.

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 separately from each other (Fig. 6).

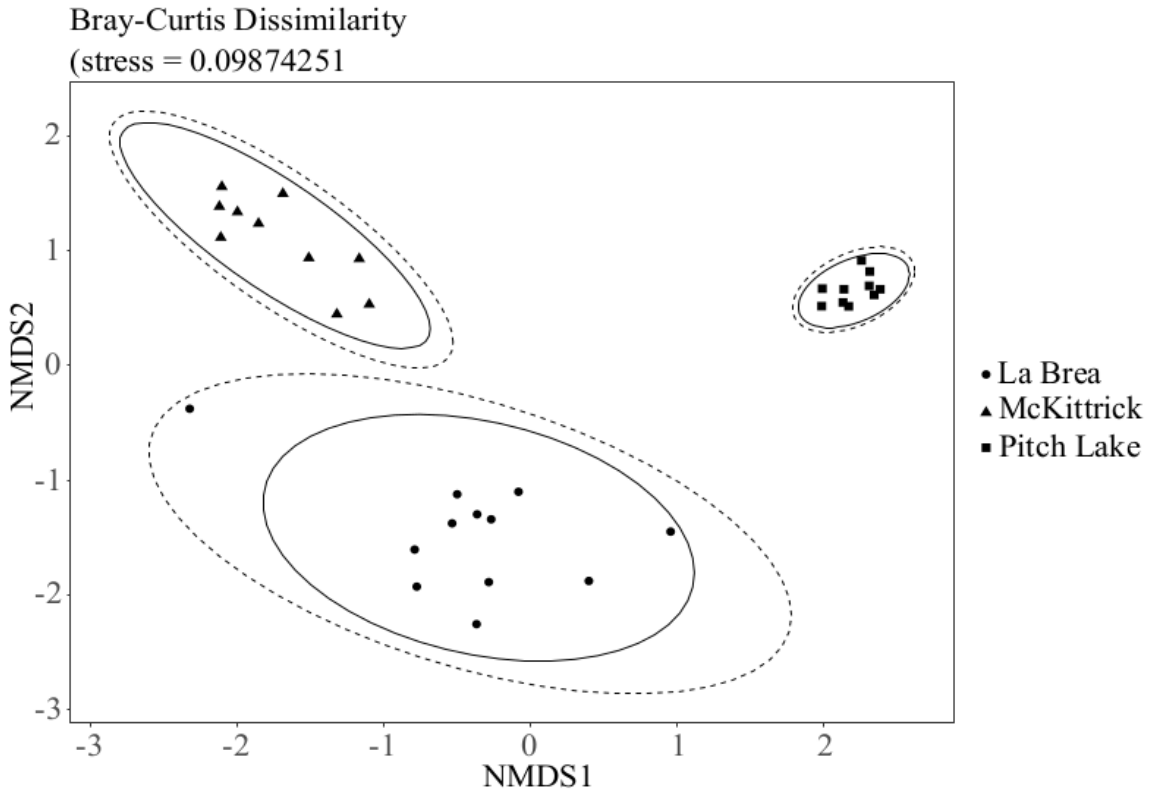


FIG 6 Nonmetric multidimensional scaling (NMDS) plot of beta diversity of all 32 water droplet communities from the three oil seeps. NMDS was calculated based on Bray-Curtis dissimilarity indices with stress level of 0.098. Dashed ellipses display the normal distribution, and solid ellipses display the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

Among the 558 OTUs identified in the three oil seeps investigated, 88 OTUs (16%) were found in all three oil seeps. Furthermore, 8 of these were present in 97 to 100% of the analyzed droplets, building a significant core community. This core community covered relative abundances 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 had 11 OTUs (2%) in common. Even though many OTUs were present in all three oil seeps, the relative abundances 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 unique OTUs (14%), and La Brea 147 unique OTUs (26%). The top 10 OTUs

of each individual droplet based on the family level are shown in Fig. S1 in the supplemental material.

4.2.3 DISCUSSION

The discovery of microorganisms in tiny water droplets suspended in oil of Pitch Lake in Trinidad, 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 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 Pitch Lake in Trinidad, the McKittrick oil field in California, and the La Brea Tar Pits in Los Angeles, CA, to look for such water droplets. Furthermore, we aimed at characterizing the principal structures of the microbial communities in this extreme environment.

Indeed, similar small water droplets of 1 to 10 µl in size were found in all three natural oil seeps. Cell counting of the suspended microorganisms in the droplets indicated dense populations up to 1.2×10^6 cells μl^{-1}. This is an astonishing density compared to other deep subsurface habitats that only contain, for example, a thousand-fold fewer cells with around 10^5 to 10^6 cells cm^{-3} (corresponding to 10^2 to 10^3 cells μl^{-1}), depending on the depth (1). With densities of 1×10^4 to 4.25×10^4 cells ml^{-1}, the microbial abundance in production water from oil reservoirs is also much lower than in our droplets (9, 18). 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 (19, 20). 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 (C8 to C28) and *n*-alcohols (C12 and C16) by sorption of hydrocarbons to extrapolymeric 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 LIVE/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 (21). With approximately 1.47×10^{-21} mol ATP per cell, the microorganisms in the water droplets contained small quantities of ATP, indicating very little activity compared to the levels in other environmental habitats, which ranged from 10^{-21} to 10^{-}

^{15} mol ATP per cell (21–23). Since ATP is rapidly consumed in the presence of biomass, we conclude that the ATP detected stemmed from living cells (21, 23–25). Hence, the results of LIVE/DEAD staining and the ATP determination indicate that the microorganisms detected in the water droplet were alive and active and not only dead microbes that were accidentally entrapped in the droplets.

The microbial community compositions showed similarities in the three oil seeps tested, and the calculated low Shannon-Wiener and high Simpson diversity indices are similar to those of other oil field microbial communities (26–29). Such values may reflect high specialization and long isolation of the communities, 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 than 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 also shared 16% of all detected OTUs with the Pitch Lake droplets. These commonalities between the three different seeps support the paradigm of Baas-Becking, “everything is everywhere, but the environment selects” (30, 31), 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 (32, 33), 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 facultatively anaerobic members, which were also reported for other oil reservoirs all over the world at mesophilic to thermophilic conditions (5–7, 34–56).

The finding that water droplets populated with active microbial communities are found in the three oil seeps tested 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 indicate that this microbial life is highly adapted.

4.2.4 MATERIALS AND METHODS

Oil sampling. Natural asphalt and heavy oil (14, 57–59) were sampled from Pitch Lake (10°14'0.6882"N, 61°37'44.5638"W) on the island of Trinidad in Trinidad and Tobago, the La Brea Tar Pits (34°03'49.7"N 118°21'25.1"W) in Los Angeles, CA, USA, and an unnamed oil seep (35°17'35.2"N 119°38'10.5"W) on the McKittrick oil field, CA, USA. The distances are 180 km between La Brea and McKittrick, 6,322 km between La Brea and Pitch Lake, and 6,462 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 samples at Pitch Lake, 30 samples at McKittrick, and 12 samples at La Brea), flushed on site directly after sampling with N_2 (5.0 grade; obtained from Massy Gas Products, Savonetta Estate, Trinidad and Tobago, for Pitch Lake and from Tyms, Inc., Los Angeles, CA, USA, for La Brea and McKittrick), and closed with a sterile gastightsealed 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, OR, USA) by pipetting directly into visible water droplets, thereby increasing the original droplet volume. 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 an HC PL APO 63×/1.4 numeric aperture (NA) 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 the 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, cell damage due to heating was 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 MΩ cm water resistivity using a Milli-Q Advantage A10 device equipped with a Q-GardT2 filter, a QuantumTEX filter, and a MillipakExpress 40 0.22-µm filter; Merck Millipore, Germany). Cells were counted with a light microscope (DMLS; Leica, Germany) equipped with a 40×/0.65 NA ocular (C Plan; Leica, Germany) and with a counting chamber (Thoma; Brand GmbH + Co. KG, Germany). In total, 10 droplets from each oil seep were examined.

To validate the first counting, an 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 (Isopore; EMD Millipore, 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, Gottingen, Germany) equipped with a 100×/1.25 NA oil objective (N-Achroplan; Carl Zeiss Microscopy GmbH, Gottingen, Germany).

Determination of cell membrane integrity in individual droplets. The membrane integrity of cells isolated from water droplets was investigated with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR, 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 being in their division cycle, leading to an overestimation of membrane-damaged cells (60–63). 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 Pitch Lake (Table S1). 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, McKittrick, and La Brea Tar Pits were diluted in 1ml of substratefree freshwater medium (64) (for Pitch Lake) or phosphate-buffered saline (pH 7.5) (for McKittrick and La Brea). Then, 3 µl of staining reagent was added to each droplet, followed by incubation for 20 min at room temperature in the dark. For dead controls,

approximately 15 µl droplet water was pooled and two 2-µl amounts of the mixture were each diluted in 1ml 70% isopropanol (BioReagent for molecular biology; Sigma-Aldrich, St. Louis, MO, USA). The controls were incubated for 1 to 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 (Isopore; EMD Millipore, 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, Gottingen, Germany) was equipped with a 100×/1.3 NA Plan-NeoFluar oil objective, LP 385 and LP 650 filters, a 488-nm argon laser, and LSM 510 software; the TCS SP8 HCS A microscope (Leica Microsystems, Germany) was equipped with an HC PL APO 63×/1.4 NA CS2 oil objective, a 488-nm and 514-nm argon laser, and LAS.X software (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 the Bio-Formats plug-in (version 5.8.2) was used for analysis.

ATP quantification. ATP (ATP) in the isolated water droplets was quantified with the BacTiter-Glo microbial cell viability assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. From each isolated droplet, 3 µl was diluted in 97 µl water and mixed with 100 µl BacTiter-Glo reagent. After 5 min of incubation, all samples were measured with a luminometer (Glomax 20/20 luminometer; Promega, Sunnyvale, CA, 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 × *g* (5810 R centrifuge; Eppendorf, Hamburg, Germany). After centrifugation, approximately 200 µl of water could be extracted from the approximately 60 ml of oil and mixed with 200 µl of BacTiter-Glo reagent (Promega, Madison, WI, USA). The solution was incubated overnight for full ATP removal. For luciferase inactivation, the mixture was heated twice for 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 MΩ cm, 3% final concentration [vol/vol], equivalent to sample volume). The processed matrix water served as the solvent for 10 mM ATP (Promega, Madison, USA), used as a reference standard.

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 data published in reference 65. In order to lyse Gram-negative bacteria and archaea, 2 µl of alkaline solution was 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) (66). 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 number 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 an 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-3`) and an overhang adaptor (5`-GTC TCG 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 (67, 68). Amplicon PCRs were performed in reaction mixture volumes of 25 µl, each containing 2 µl of extracted DNA, 12.5 µl of 2× KAPA HiFi hot start ready mix (KAPA Biosystems, MA, USA), and 0.25 µM 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 to 55°C, with a decline of 0.5°C per cycle, and 30 s at 72°C, continuing with 30 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 10 min. Amplicon PCR products were checked by agarose gel electrophoresis with 1% (wt/vol) 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 version 2 set D (FC-131-2004; Illumina, 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. The DNA concentration of each sample was quantified using the Qubit double-stranded DNA (dsDNA) high-sensitivity (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 (version 1.40.5, last updated 19 June 2018) MiSeq standard operating procedure (SOP) (69, 70). After merging forward and reverse reads, sequences with ambiguous bases, shorter than 380 bp or longer than 470 bp, were removed from the data set. All remaining unique sequences were aligned to the bacterial database SILVA, version 132, customized to the region of interest (71–73). Chimeras and nonribosomal sequences were removed and taxonomic classification was assigned based on RDP, trainset 16 (Ribosomal Database Project) (74). Sequences were clustered into operational taxonomic units (OTUs) by defining a 97% similarity cutoff (setting of 0.03 distance limit). Reads were rarified via mothur to the lowest detected read number of 14,281 of sample 46_PL (Pitch Lake). The R package phyloseq (75) 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 mistakes and removed from the data set. Afterwards, technical replicates were pooled by calculating the mean number of reads for each OTU.

Data availability. Raw sequencing reads were deposited in the NCBI database in BioProject under accession number PRJNA546121.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

This work was supported by the European Research Council (grant number 666952-EcOILogy) and the German Research Foundation (grant number BR 5493/1-1).

We thank the La Brea Tar Pit and Museum and especially Aisling Farrell and Gary Takeuchi for access to the La Brea sampling spots and support during the sampling. We thank Jeffrey Prude from the Bakersfield Field Office Oil and Gas Program Lead for information and help with accessing the area, as well as the Chevron Corporation for sampling permission. We also thank David West from the California State Parks, Channel Coast District, for permission and support during sampling. Further thanks go to Nina Schulze and Johannes Koch from the Imaging Centre Campus Essen of the Center of Medical Biotechnology, University of Duisburg-Essen, Germany for providing the CLSM. Additional thanks to Alexander Probst for providing computing power for bioinformatics calculations.

The authors report no competing financial interests.

4.2.5 REFERENCES

1. Head IM, Jones DM, Larter SR. 2003. Biological activity in the deep subsurface and the origin of heavy oil. Nature 426:344–352. https://doi.org/10.1038/nature02134.

2. Aitken CM, Jones DM, Larter S. 2004. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature 431:291–294. https://doi.org/10.1038/nature02922.

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. https://doi.org/10.1016/S0146-6380(02)00240-1.

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. https://doi.org/10.1016/j.orggeochem.2012.12.011.

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 6:20174. https://doi.org/10.1038/srep20174.

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. https://doi.org/10.1016/j.petrol.2014.07.030.

7. Magot M, Ollivier B, Patel B. 2000. Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 77:103–116. https://doi.org/10.1023/A:1002434330514.

8. Pannekens M, Kroll L, Muller H, Mbow FT, Meckenstock RU. 2019. Oil reservoirs, an exceptional habitat for microorganisms. New Biotechnol 49:1–9. https://doi.org/10.1016/j.nbt.2018.11.006.

9. Lenchi N, I˙nceog˘ lu O, Kebbouche-Gana S, Gana ML, Lliros 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:e66588. https://doi.org/10.1371/journal.pone.0066588.

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 Biodeterior Biodegradation 120:170–185. https://doi.org/10.1016/j.ibiod.2017.02.005.

11. Youssef N, Elshahed MS, McInerney MJ. 2009. Microbial processes in oil fields: culprits, problems, and opportunities. Adv Appl Microbiol 66:141–251. https://doi.org/10.1016/S0065-2164(08)00806-X.

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. Document SPE-24811-MS. *In* SPE Annual Technical Conference and Exhibition. Society of Petroleum Engineers, Richardson, TX. https://doi.org/10.2118/24811-MS.

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. https://doi.org/10.1038/srep16057.

14. Kim JS, Crowley DE. 2007. Microbial diversity in natural asphalts of the Rancho La Brea tar pits. Appl Environ Microbiol 73:4579–4591. https://doi.org/10.1128/AEM.01372-06.

15. Meckenstock RU, von Netzer F, Stumpp C, Lueders T, Himmelberg AM, Hertkorn N, Schmitt-Kopplin P, Harir M, Hosein R, Haque S, Schulze-Makuch D. 2014. Water droplets in oil are microhabitats for microbial life. Science 345:673–676. https://doi.org/10.1126/science.1252215.

16. Head I, Larter S, Gray N, Sherry A, Adams J, Aitken C, Jones D, Rowan A, Huang H, Roling W. 2010. Hydrocarbon degradation in petroleum reservoirs, p 3097–3109. *In* Timmis KN, McGenity TJ, Roelof van der Meer J, de Lorenzo V (ed), Handbook of hydrocarbon and lipid microbiology.Springer-Verlag, Berlin, Germany.

17. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schaffer AA. 2008. Database indexing for production MegaBLAST searches. Bioinformatics 24:1757–1764. https://doi.org/10.1093/bioinformatics/btn322.

18. 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 66:700–711. https://doi.org/10.1128/AEM.66.2.700-711.2000.

19. 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. https://doi.org/10.1002/bit.22577.

20. Johnsen AR, Karlson U. 2004. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. Appl Microbiol Biotechnol 63:452–459. https://doi.org/10.1007/s00253-003-1265-z.

21. Shama G, Malik DJ. 2013. The uses and abuses of rapid bioluminescencebased ATP assays. Int J Hyg Environ Health 216:115–125. https://doi.org/10.1016/j.ijheh.2012.03.009.

22. 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. https://doi.org/10.1016/j.watres.2010.04.015.

23. 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. https://doi.org/10.1016/0022-0981(81)90049-6.

24. Cowan DA, Casanueva A. 2007. Stability of ATP in Antarctic mineral soils. Polar Biol 30:1599–1603. https://doi.org/10.1007/s00300-007-0324-9.

25. Karl DM. 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol Rev 44:739–796. https://doi.org/10.1128/MMBR.44.4.739-796.1980.

26. Xiao M, Zhang Z-Z, Wang J-X, Zhang G-Q, 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. Bioresour Technol 147:110–116. https://doi.org/10.1016/j.biortech.2013.08.031.

27. 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, Wu W-M. 2016. Analysis of bacterial diversity in two oil blocks from two low-permeability reservoirs with high salinities. Sci Rep 6:19600. https://doi.org/10.1038/srep19600.

28. Silva T, Verde L, Neto ES, Oliveira V. 2013. Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. Int Biodeterior Biodegradation 81:57–70. https://doi.org/10.1016/j.ibiod.2012.05.005.

29. 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-insolubleparticle and formation-water components of the reservoir fluid from a non-flooded high-temperature petroleum reservoir. J Biosci Bioeng 113:204–210. https://doi.org/10.1016/j.jbiosc.2011.09.015.

30. Fondi M, Karkman A, Tamminen MV, Bosi E, Virta M, Fani R, Alm E, McInerney 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. https://doi.org/10.1093/gbe/evw077.

31. Baas-Becking L. 1934. Geobiologie; of inleiding tot de milieukunde. WP Van Stockum & Zoon NV, The Hague, The Netherlands.

32. Shade A, Stopnisek N. 2019. Abundance-occupancy distributions to prioritize plant core microbiome membership. Curr Opin Microbiol 49:50–58. https://doi.org/10.1016/j.mib.2019.09.008.

33. Sieber M, Pita L, Weiland-Brauer N, Dirksen P, Wang J, Mortzfeld B, Franzenburg S, Schmitz RA, Baines JF, Fraune S, Hentschel U, Schulenburg H, Bosch TCG, Traulsen A. 2019. Neutrality in the metaorganism. PLoS Biol 17:e3000298. https://doi.org/10.1371/journal.pbio.3000298.

34. Orlygsson J, Kristjansson JK. 2014. The family Hydrogenophilaceae, p 859–868. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: alphaproteobacteria and betaproteobacteria. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-30197-1_244.

35. 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 44:145–153. https://doi.org/10.2134/jeq2014.04.0164.

36. 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. https://doi.org/10.1007/s10295-014-1401-z.

37. 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. https://doi.org/10.1099/ijs.0.02538-0.

38. Sakamoto M. 2014. The family Porphyromonadaceae, p 811–824. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: other major lineages

of bacteria and the archaea. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-38954-2_132.

39. 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. https://doi.org/10.1016/j.femsec.2005.05.007.

40. Willems A, Gillis M. 2005. *Comamonadaceae* Willems, De Ley, Gillis and Kersters 1991a, 447VP, p 686–688. *In* Brenner DJ, Krieg NR, Staley JT (ed), Bergey's manual of systematic bacteriology, vol 2, part C. Springer, New York, NY.

41. Willems A. 2014. The family Comamonadaceae, p 777–851. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: alphaproteobacteria and betaproteobacteria. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-30197-1_238.

42. 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. https://doi.org/10.1099/00207713-49-2-567.

43. Willems A, Pot B, Falsen E, Vandamme 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. https://doi.org/10.1099/00207713-41-3-427.

44. Perez-Pantoja D, Donoso R, Agullo L, Cordova M, Seeger M, Pieper DH, Gonzalez B. 2012. Genomic analysis of the potential for aromatic compounds biodegradation in *Burkholderiales*. Environ Microbiol 14:1091–1117. https://doi.org/10.1111/j.1462-2920.2011.02613.x.

45. Oren A. 2014. The family Methanotrichaceae, p 297–306. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: other major lineages of bacteria and the archaea. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-38954-2_277.

46. 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. https://doi.org/10.1627/ jpi.52.297.

47. Kuever J. 2014. The family *Desulfobulbaceae*, p 75–86. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: deltaproteobacteria and

epsilonproteobacteria. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-39044-9_267.

48. Kuever J. 2014. The family *Syntrophobacteraceae*, p 289–299. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The prokaryotes: deltaproteobacteria and epsilonproteobacteria. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-39044-9_268.

49. Galushko A, Kuever J. 2019. Desulfoprunum. *In* Whitman WB, deVos P, Dedysh S, Hedlund B, Kampfer P, Rainey F, Trujillo ME, Bowman JP, Brown DR, Glockner FO, Oren A, Paster BJ, Wade W, Ward N, Busse H-J, Reysenback A-L (ed), Bergey's manual of systematics of archaea and bacteria. Springer, New York, NY. https://doi.org/10.1002/9781118960608.gbm01593.

50. 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. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, vol 1. Springer, New York, NY.

51. 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. https://doi.org/10.1099/ijsem.0.002234.

52. 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. https://doi.org/10.1099/00207713-47-1-150.

53. 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. https://doi.org/10.1007/s10482-013-0062-7.

54. 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. https://doi.org/10.1007/s10482-007-9177-z.

55. Sherry A, Gray N, Ditchfield A, Aitken C, Jones D, Roling 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 Biodeterior Biodegradation 81: 105–113. https://doi.org/10.1016/j.ibiod.2012.04.009.

56. Liang B, Wang L-Y, Mbadinga SM, Liu J-F, Yang S-Z, Gu J-D, Mu B-Z. 2015. *Anaerolineaceae* and *Methanosaeta* turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. AMB Express 5:117. https://doi.org/10.1186/s13568-015-0117-4.

57. Meyer RF, Attanasi ED. 2003. Heavy oil and natural bitumen—strategic petroleum resources. Fact sheet 70-03. US Geological Survey, US Department of the Interior, Reston, VA. https://pubs.er.usgs.gov/publication/fs07003.

58. Hein FJ. 2013. Overview of heavy oil, seeps, and oil (tar) sands, California, p407–435. *In* Hein FJ, Leckie D, Larter S, Suter JR (ed), Heavy-oil and oil-sand petroleum systems in Alberta and beyond: AAPG studies in geology, vol 64. American Association of Petroleum Geologists, Tulsa, OK.

59. 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. https://doi.org/10.1089/ast.2010.0488.

60. Frosler J, Panitz C, Wingender J, Flemming H-C, Rettberg P. 2017. Survival of Deinococcus geothermalis in biofilms under desiccation and simulated space and Martian conditions. Astrobiology 17:431–447. https://doi.org/10.1089/ast.2015.1431.

61. 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. https://doi.org/10.1128/AEM.68.11.5209-5216.2002.

62. Davey HM, Hexley P. 2011. Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. Environ Microbiol 13:163–171. https://doi.org/10.1111/j.1462-2920.2010.02317.x.

63. Shi L, Gunther S, Hubschmann T, Wick LY, Harms H, Muller S. 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry 71A:592–598. https://doi.org/10.1002/cyto.a.20402.

64. Widdel F, Pfennig N. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Arch Microbiol 129:395–400. https://doi.org/10.1007/BF00406470.

65. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. 2012. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 7:e33865. https://doi.org/10.1371/journal.pone.0033865.

66. 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. https://doi.org/10.2144/000114389.

67. 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. https://doi.org/10.1371/journal.pone.0105592.

68. Graspeuntner S, Loeper N, Kunzel 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. https://doi.org/10.1038/s41598-018-27757-8.

69. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: opensource, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09.

70. 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. https://doi.org/10.1128/AEM.01043-13.

71. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41: D590–D596. https://doi.org/10.1093/nar/gks1219.

72. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glockner FO. 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res 42: D643–D648. https://doi.org/10.1093/nar/gkt1209.

73. Glockner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, Bruns G, Yarza P, Peplies J, Westram R, Ludwig W. 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol 261:169–176. https://doi.org/10.1016/j.jbiotec.2017.06.1198.

74. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. https://doi.org/10.1128/AEM.00062-07.

75. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217. https://doi.org/10.1371/journal.pone.0061217.

4.2.6 Supplemental Material - Densely Populated Water Droplets in Heavy-Oil Seeps

Community composition per droplet

Typical microbial inhabitants of oil reservoirs were found in all water droplets.

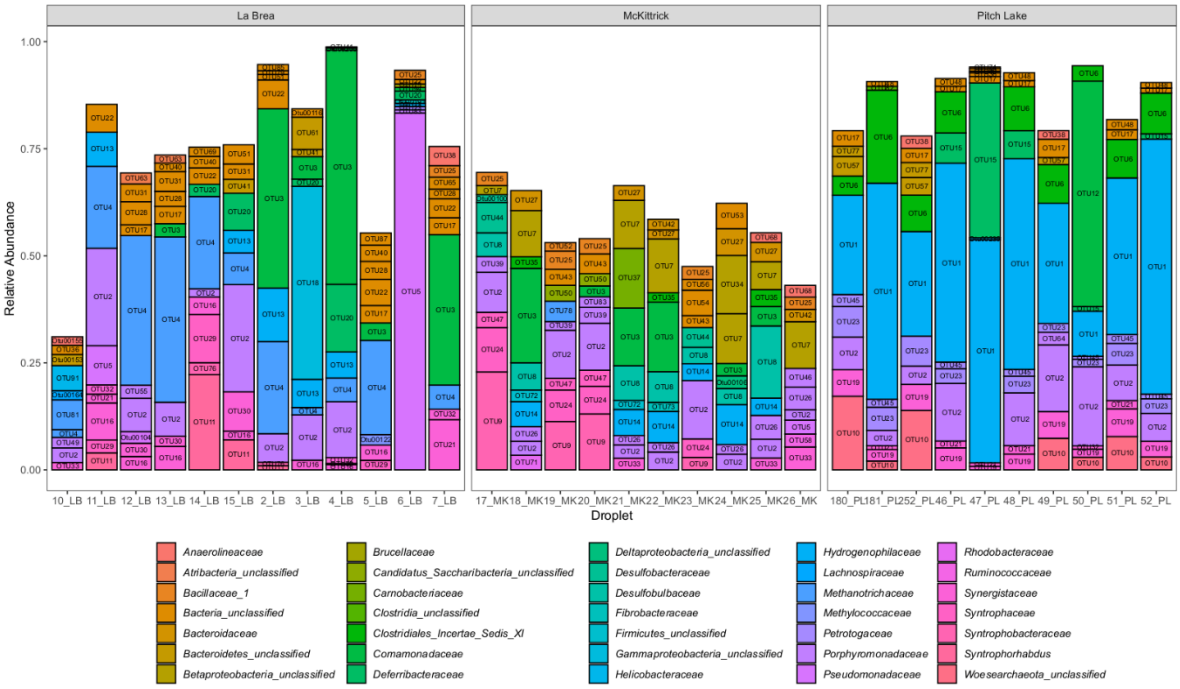


Fig. S1. The 10 most abundant OTUs per individual droplet community. Boxes represent OTUs colored by family.

LIVE/DEAD-Staining method validation

To avoid false negative staining results due to overstaining of propidium iodide, the manufacturer's instructions were modified according to Frösler et al., 2017 (1). Different propidium iodide concentrations (shown in table S1) were tested and a sulfate-reducing, phenanthrene-degrading mixed culture isolated from the Pitch Lake served as positive control. A culture treated with 70 % isopropanol (99.5 % for molecular biology; Sigma-Aldrich, St. Louis, USA) for 1 h, served as dead control.

Table S1 shows the tested propidium iodide concentrations for method validation

Syto®9/PI mixed in ratio 1:1				
	Test 1	Test 2	Test 3	Test 4
Syto®9	3.34 mM	3.34 mM	3.34 mM	3.34 mM
PI	18.3 mM	0.366 mM	0.183 mM	0.092 mM

REFERENCES

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

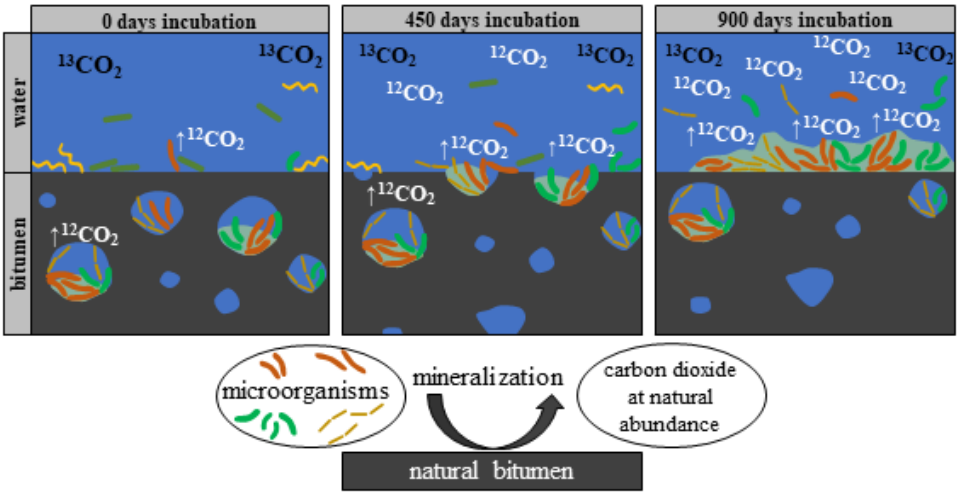
4.3 Microbial degradation rates of natural bitumen

Mark Pannekens, Lisa Voskuhl, Sadjad Mohammadian, Daniel Köster, Arne Meier, John. M. Köhne, Michelle Kulbatzki, Ali Akbari, Shirin Haque, Rainer U. Meckenstock*

Cite This: Environ. Sci. Technol. 2021, 55, 8700−8708
Received: January 27, 2021
Revised: June 10, 2021
Accepted: June 15, 2021
Published: June 25, 2021
https://doi.org/10.1021/acs.est.1c00596

ABSTRACT: Microorganisms are present in nearly every oil or bitumen sample originating from temperate reservoirs. Nevertheless, it is very difficult to obtain reliable estimates about microbial processes taking place in deep reservoirs, since metabolic rates are rather low and differ strongly during artificially cultivation. Here, we demonstrate the importance and impact of microorganisms entrapped in microscale water droplets for the overall biodegradation process in bitumen. To this end, we measured degradation rates of heavily biodegraded bitumen from the Pitch Lake (Trinidad and Tobago) using the novel technique of reverse stable isotope labeling, allowing precise measurements of comparatively low mineralization rates in the ng range in microcosms under close to natural conditions. Freshly taken bitumen samples were overlain with artificial brackish water and incubated for 945 days. Additionally, three-dimensional distribution of water droplets in bitumen was studied with computed tomography, revealing a water bitumen interface of 1134 cm^2 per liter bitumen, resulting in an average mineralization rate of 9.4−38.6 mmol CO_2 per liter bitumen and year. Furthermore, a stable and biofilm-forming microbial community established on the bitumen itself, mainly composed of fermenting and sulfate-reducing bacteria. Our results suggest that small water inclusions inside the bitumen substantially increase the bitumen−water interface and might have a major impact on the overall oil degradation process.

KEYWORDS: *asphalt, oil reservoirs, micro habitats, water droplets, isotope dilution, anaerobic biodegradation, petroleum mineralization, biofilm formation*



4.3.1 INTRODUCTION

Crude oil is one of the most important natural resources in our modern life but a major fraction of economically available oil exists as biodegraded heavy oil or bitumen.[1–3] During the degradation process, *n*-alkanes, monocyclic alkanes, and alkyl benzenes disappear first, which leads to a lower oil quality and finally to natural bitumen mostly consisting of saturated and aromatic hydrocarbons, resins, and asphaltene.[4–6] However, our knowledge about *in situ* biological processes and degradation rates in anoxic oil reservoirs is scarce due to the limited access to the deep subsurface.[1,2] The most common metabolisms found in oil reservoirs are sulfate reduction, fermentation, acetogenesis, and methanogenesis.[3,5,7,8] A broad range of metabolic pathways like iron(III)-, manganese(IV)-, nitrate-, or nitrite reduction have been detected in oil reservoirs as well.[9,10] Nevertheless, their influence still remains unclear, since the microbial degradation of hydrocarbons in the environment is mostly limited by the availability of electron acceptors and the bioavailability of the hydrocarbons.[3,11] Since electron acceptors in oil reservoirs are quickly depleted by microorganisms and do not get replenished unless water flooding is implemented, fermentation and methanogenesis are believed to be mainly responsible for the oil degradation in most reservoirs.[9,10,12] Fermentative, syntrophic microorganisms degrade complex hydrocarbons like fatty acids, alcohols, sugars, amino acids, and aromatic compounds stepwise into smaller molecules such as hydrogen, acetate, and carbon dioxide.[12–17] In addition, acetate is alternatively oxidized by syntrophic acetate oxidation.[15–17] Many of these reactions are endergonic and are only energetically feasible if the products are kept at low concentrations.[12,16] Acetate is commonly converted by acetoclastic methanogens into methane and carbon dioxide, whereas hydrogen and carbon dioxide are used by hydrogenotrophic methanogens to produce methane.[12–17]

However, sulfate-reducing bacteria (SRB) are present in practically every oil reservoir and are known for complete alkane (C_3–C_{20}) oxidation to carbon dioxide in the presence of sulfate.[7,18] They are also capable of mineralizing aromatic hydrocarbon degradation such as benzene, toluene, naphthalene, 2-methylnaphthalene, phenanthrene, ethylbenzene, and xylene carbon dioxide.[5,18−20]

Even if forced in experimental setups, anaerobic biodegradation of oil is an extremely slow process which is difficult to assess. In the natural environment, biodegradation is assumed to be even slower or sometimes completely inhibited due to temperature and other abiotic factors. Therefore, we applied the relatively new method of reverse stable isotope labeling, enabling the measurement of degradation of unknown carbon sources even at extremely low rates.[21−24] This robust and sensitive method is based on the dilution of a ^{13}C-labeled bicarbonate buffer with nonlabeled carbon dioxide from biodegradation of hydrocarbons at natural isotopic abundance.[21]

The second major limitation for biodegradation of hydrocarbons is the poor bioavailability (i.e., water solubility and dissolution kinetics). Hence, biodegradation rates depend to a large extent on the mass transfer of hydrocarbons into the aqueous phase and the surface area of the oil−water interface is a limiting factor for oil degradation. This is supported by the common concept of biodegradation in crude oil reservoirs, indicating that microbial degradation takes place at the bottom of the oil-bearing leg at the oil−water-transition-zone and is, thus, limited by the interface area.[25] However, it was discovered that bitumen contains small water droplets and pockets populated with oil-degrading microbial communities.[26−28] Assuming that the area of the oil−water interface is a limiting factor for biodegradation in oil reservoirs, such water droplets might potentially increase the overall degradation kinetics by increasing the total oil−water surface area. When the transfer of hydrocarbons into the water phase is rate-limiting, microorganisms often accumulate at the substrate surface, here the oil−water interface, to overcome the transfer limitations. Several studies showed the formation of biofilms in the presence of oil, oil sands, or bitumen when incubated in artificial media containing additives like vitamins or trace elements with pure- or enrichment cultures.[29−32]

The aim of this study was to determine the impact of microbially populated microscale water droplets enclosed in natural bitumen on the overall biodegradation process. Therefore, surface dependent biodegradation rates of bitumen were analyzed and combined with size and distribution measurements of enclosed water droplets in bitumen. Our findings allowed us to calculate a theoretical degradation rate per intra bitumen interface area and indicate a notable

amplification of the bitumen−water transition zone through microscale water droplets in bitumen phase.

4.3.2 MATERIALS AND METHODS

Bitumen Smpling. Bitumen was sampled from the Pitch Lake (N 10°14′0.6882″, W 61°37′44.5638″) located in Trinidad, Trinidad and Tobago. Liquid bitumen freshly seeping on the Pitch Lake was sampled with decapitated 50 mL syringes and directly transferred into sterile microcosms. To avoid oxygen-contaminated samples, the bitumen samples were taken from 10 to 15 cm beneath the surface, after the spring was refilled by fresh bitumen from the subsurface. The microcosms were then flushed directly on site with N_2 (5.0 grade; Massy Gas Products, Trinidad and Tobago) and sealed with butyl stoppers. After shipping, each microcosm was flushed again for 15 min with N_2 (5.0 grade; sterile through upstream filters). The surface temperature of the sampled bitumen was ∼33.5 °C as measured on site with a PL-120-digital thermometer T2 (Voltcraft, Switzerland). The four microcosms 1−4 contained 109.03, 116.39, 64.42, and 130.03 g bitumen, respectively. Additional bitumen from the same seep was sampled in sterile glass jars for further analysis.

Microcosm Setup. To analyze the geochemistry of the enclosed water droplets, bitumen from additional glass jars was heated to 40−45 °C for ∼30 min. Aliquots of ∼15 g were transferred into 50 mL centrifugation tubes and centrifuged for 120 min at 3214 g. The water on top of the bitumen was collected and the procedure was repeated until no water appeared after centrifugation. The anion and cation contents of the water were determined by ion chromatography (Dionex, CA, U.S.A.; Metrohm, Filderstadt, Germany) (for detailed sample preparation and measurement conditions see S1 of the Supporting Information, SI). The pH of extracted water was 7.9.

On the basis of the geochemical composition of the droplet water (see S2), an anoxic brackish water was prepared with a final concentration of 16.3 mM NH_4Cl, 5.4 mM KCl, 1.1 mM $CaCl_2{\cdot}2H_2O$, 1.3 mM $MgCl_2{\cdot}6H_2O$, 173 mM NaCl, 9.7 mM $NaHSO_4$, and 31.7 mM $NaH_2PO_4{\cdot}2H_2O$. In addition, 1 mM resazurin was added as redox indicator. Then, the brackish water was boiled to remove O_2 and CO_2. Subsequently, the bottle was sealed with a butyl stopper, flushed with Biogon (CO_2/N_2 = 20/80 (v/v); Air Liquide, Germany) and autoclaved. Na_2S (sterile, anaerobic) was added as reducing agent to a final concentration of 0.5 mM (according to Widdel et al., 1981) to ensure an oxygen free environment.[33] The brackish medium was buffered with a $NaHCO_3$ buffer solution (sterile, anaerobic; final concentration 30 mM) and adjusted to pH 7, after injection into the microcosms the pH rose

to ∼8 (close to the natural measured 7.9). The carbonate buffer was ^{13}C-labeled with $x(^{13}C)$ = 10 atom % as a mixture of regular $NaHCO_3$ $x(^{13}C)$ = 1.11% (Carl Roth, Germany) and ^{13}Clabeled $NaHCO_3$ $x(^{13}C)$ = 98% (Sigma-Aldrich, MO, U.S.A.).[33] In total, 140 mL of the buffered and reduced brackish water was added without further supplements to each bitumen-containing microcosms 1−4 to artificially increase the present natural water volume. All microcosms were incubated at ∼32 °C.

Bitumen sterilization: In contrast to aerobic, abiotic bitumen oxidation (<125−135 °C), anoxic thermal alteration of bitumen starts to be quantitatively relevant at temperatures >200 °C. However, there are strong indications that decomposition of minor fraction occurs even at temperatures <200 °C.[34−37] Most of the bitumen (∼90%) boils at temperatures >350 °C, nonetheless, even much lower temperatures (40−120 °C) strongly affect the bitumen viscosity which decreases while heating.[38−41] A lower viscosity enables entrapped inorganic compounds with low boiling points, such as carbon dioxide, carbon monoxide, hydrogen sulfide, or carbonyl sulfide and smaller organic compounds present in bitumen such as neopentane, methane, acetaldehyde, propane, propylene, pyridine, and benzene, to degas from the bitumen.[37,42−44] Therefore, autoclaving the bitumen likely leads to compound decompositions and changes of the matrix and certainly to a degassing of various compounds necessary for microbial growth. Another frequently used method for sterilization is γ radiation. However, several studies showed that bitumen is affected by γ radiation, resulting in radiolytic gas production (hydrogen) and swelling of the bitumen body causing changes of the overall composition.[45−48] Chemical sterilization would also affect and change the bitumen matrix. Additionally, spore formers with unknown germination times are present in many oil fields which are difficult to monitor within the bitumen.[49−51] Because of these unknown matrix effects, we did not setup sterile controls.

Assessing Bitumen Mineralization. The parameters carbon dioxide development, cell density, sulfate concentration, and pH were monitored during the 945 days of incubation at days 0, 42, 93, 140, 259, 470, 604, 877, and 945. All samples were taken anaerobically with N_2 flushed syringes (5.0 grade; sterile Hungate needle).

For analyzing CO_2-development, aqueous samples (0.5 mL) were taken with syringes through the stoppers and directly transferred into 12 mL Labco Exertainer vials (Labco Limited,U.K.) which were preamended with 100 μL of 85% phosphoric acid, closed with screw caps containing butyl rubber septa, and flushed with CO_2-free synthetic air (6.0 grade; Air Liquide, Germany).[21−23,52] Samples were analyzed with a Delta Ray CO_2 Isotope Ratio Infrared Spectrometer (Thermo Fisher Scientific, MA, U.S.A.) with Universal Reference

Interface Connect for measuring carbon isotope compositions of CO_2.[21−23,53] CO_2-free synthetic air (Air Liquide, Germany) was used as carrier gas. CO_2 in synthetic air at 414.2 ppm (Air Liquide, Germany) was used for CO_2 concentration calibration. CO_2 reference gases used for calibration of carbon isotope ratios had $\delta^{13}C$ values of −9.7‰ (Thermo Fisher, Bremen, Germany) and $x(^{13}C)$ = 10% (Sigma-Aldrich, Taufkirchen, Germany). Pure CO_2 gas with $x(^{13}C)$ = 10% was used as working reference gas. The CO_2 concentration for reference and sample gas entering the analyzer was set to 380 ppm for optimal precision. Each sample was measured for 5 min and the obtained $\delta^{13}C$ values were averaged. The stable carbon isotope data were received as delta values and converted into isotope-amount fraction according to Coplen et al., (2011).[54]

To determine the isotope amount fraction $x(^{13}C)$ of the electron donor bitumen, 0.779, 0.861, and 0.467 mg bitumen were transferred into tin vials and measured with a Pyro Cube element analyzer (Elementar Analysensysteme, Langenselbold, Germany) using the CN mode coupled to an IsoPrime 100 isotope ratio mass spectrometer (IRMS, (Elementar Analysensysteme, Langenselbold, Germany), as described in Köster et al., (2018).[55]

Microbial growth in the aqueous phase was monitored by cell counting with light microscopy (DMLS, Leica, Germany), equipped with a 40x/0.65 ocular and a Thoma counting chamber (Brand GmbH + Co KG, Germany). Sulfate was measured with ion chromatography (IC) (see S7).

Microbial Community Composition. For DNA extraction of planktonic cells, 1.5 mL (time points 0 and 4) and 0.5 mL (time points 6 and 8) water samples were extracted with the DNeasy PowerLyser Power Soil Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions with modified steps as described in the SI (see S2).

At the end of the incubation, the remaining water was removed from each microcosm and the microcosms containing the bitumen were frozen at −70 °C for 4 h. To extract DNA from cells on the bitumen surface, thin pieces were flaked off the frozen bitumen surface with a scalpel, 0.4−0.7 g were transferred into triplicate bead-beating tubes per microcosm. Bitumen samples were extracted as described in Himmelberg (2018).[56] A further cleaning step for bitumen samples after the extraction was modified from the described purification step in the Illumina protocol to remove humic acids and other matrix components which inhibit the PCR (see S3). Amplification of the 16S rRNA genes, library preparation, and sequencing were performed as described in Pannekens et al., (2020).[27,57] Raw sequencing reads were deposited in the NCBI database under BioProject PRJNA645080.

Biofilm Visualization on Bitumen Surface. Biofilms were visualized with seven fluorescence-labeled lectins including Concanavalin A (Con A), Peanut agglutinin (PNA), Ulex Europaeus Agglutinin I (UEA), *Griffonia simplicifolia* lectin II (GS II), wheat germ agglutinin (WGA), soybean agglutinin (SBA), *Oncorhynchus* keta Lectin (CSL 3) to cover a broad range of target sugars which might be part of the extracellular polymeric substances (EPS) matrix.[58–61] The lectins (10 μg mL^{-1} each final concentration) were mixed in a buffer solution consisting of 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, 0.1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MnCl_2 \cdot 4H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, pH 7.5, and 10 μM Syto9 as counter stain for microbial cells. The staining mixture (50 μL) was added to each sample and incubated for 30 min in the dark. Afterward, the samples were gently rinsed three times with deionized water to remove the staining mix.[61] Drinking water biofilms grown on ethylene propylene diene monomer (EPDM) coupons served as staining controls. An epifluorescence microscope was used for sample analysis. For detailed information see S5.

3D Imaging of Bitumen−Droplet Distribution. The water droplet distribution was determined via quantitative image analysis of CT scans. To this end, Pitch Lake bitumen was either filled directly in the field (3 columns, filled by pulling) or in the laboratory (3 columns, transferred by syringe after incubation at 45 °C) into round plastic columns (15 cm in length with a diameter of 1.5 or 1 cm, respectively). The top and bottom were sealed with rubber stoppers in the field and exchanged with hot glue in the laboratory. The sealed columns were placed on plastic stands and stored at 4 °C until analysis. 3D images were taken using X-ray computed tomography (CT) at a spatial resolution of 12.5 μm. Quantitative image analysis was used to segment the images and quantify the size destruction of the water droplets. For detailed information on scanning condition and image analysis steps please see S9.

4.3.3 RESULTS AND DISCUSSION

Mineralization of Bitumen. Surprisingly, sulfate was present in all extracted water samples (9.7 ± 3.9 mM on average, see S2) and fermenting and sulfate-reducing bacteria were the most abundant members of the original community. Therefore, we focused mainly on bitumen mineralization to carbon dioxide by fermenting and sulfate-reducing bacteria (for detailed equations see S10), since sulfate reducers often outcompete or inhibit methanogenesis if sulfate is available in quantitative amounts.[6,12,32,62,63] Sulfate reduction and CO_2 production were monitored in four microcosms to assess the mineralization and degradation rates of bitumen. Reproducible sulfate reduction occurred in all microcosms

(Figure 1A) with 6.7, 9.5, 8.7, and 9.0 mM consumed over 945 days, respectively (average sulfate reduction of 8.5 ± 1.1 mM).

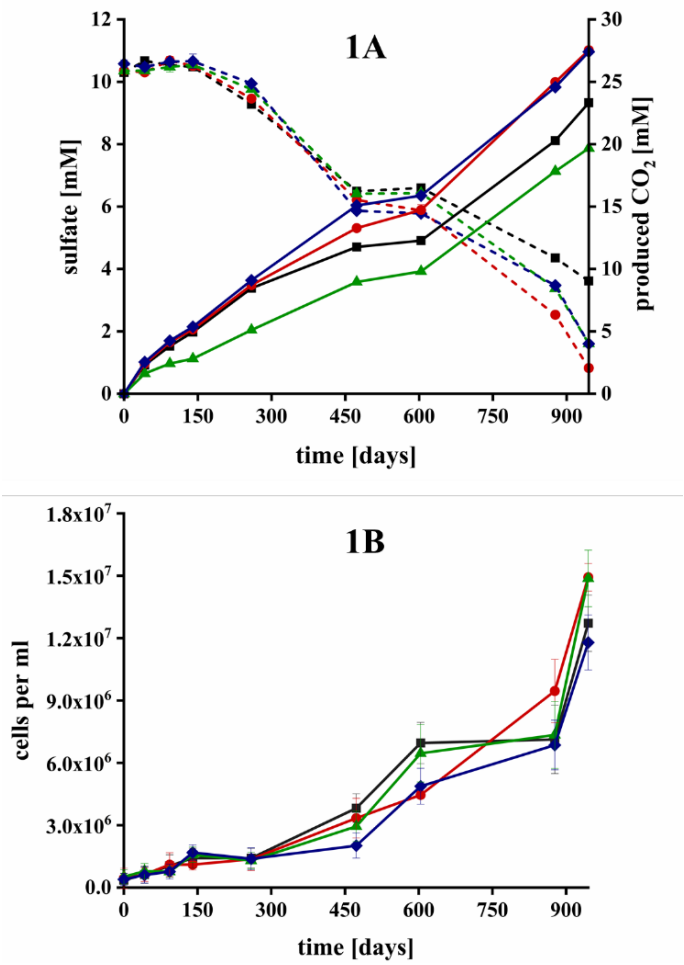


Figure 1. Degradation of bitumen in microcosms with sulfate as electron acceptor in microcosm 1 (black ■), 2 (red ●), 3 (green ▲), and 4 (blue ♦) over 945 days of incubation. (1A) Degradation was measured by formation of carbon dioxide (solid lines) and depletion of sulfate (dashed lines). Error bars depict the standard deviation of triplicate analytical measurements but are barely visible because they are smaller than the symbol size. (1B) Microbial growth was assessed with light microscopy and a Thoma counting-chamber. Error bars depict the standard deviation of a minimum of six countings per sample.

Correspondingly, the CO_2 concentrations increased by 23.3, 27.5, 19.7, and 27.4 mM, respectively, with an average production of 24.5 ± 3.2 mM CO_2 during 945 days of incubation. Considering the used water volume leads to an average absolute production of 2.93 mmol CO_2 during the incubation or 1.13 mmol CO_2 per year. The values were calculated based on the measured isotopic signature of the bitumen of $x(^{13}C)$ = 1.075%. The pH values fluctuated slightly between 7.8−8.3 during the entire incubation, which ensured that the

majority of CO_2 was dissolved as bicarbonate in the aqueous phase. Previous analyses of the bitumen composition showed that short chain alkanes and alkenes with a carbon oxidation state of -II to -III had been already depleted.[19,26] On the basis of the C/H ratio of Pitch Lake bitumen measured by Meckenstock et al., (2014), we estimated a carbon oxidation state of approximately −1.38 in the bitumen.[26] Hence, we calculated an average electron balance for the four microcosms assuming that sulfate was reduced to sulfide (8.5 mM × $8e^-$). Only ∼12.6 mM of the produced CO_2 (∼52%) could be linked to sulfate reduction based on the produced CO_2 (24.5 mM × $5.38e^-$).[26] A possible explanation are fermenters, which are present in numerous oil reservoirs and play an important role in the overall hydrocarbon degradation. Fermenters often belong to the most abundant microorganisms after incubation of oil, heavy oil, or water from oil reservoirs.[5,64−67] Among others, several members of the phylum *Chloroflexi*, the order *Desulfobacterales*, the family *Deferribacteraceae*, or the genus *Thermovirga* were present in our communities. These microorganisms are either known fermenters or possess at least the ability for hydrocarbons fermentation in oil reservoirs.[5,63−65,67−70] In addition, many sulfate reducers present in oil reservoirs can also ferment in the absence of sulfate or when sulfate reduction is inhibited.[12] Other fermenting microorganisms often live in syntrophic communities by providing metabolites such as hydrogen and acetate to microorganisms like SRB or methanogens.[8,24,49,62] Therefore, it is likely that the remaining carbon and electron equivalents were not evolved as CO_2 but were present as fermentation products.

Cell Growth and Community Evolution over Time. To assess the microbial growth in the presence of bitumen as sole carbon and electron source, we monitored the cell numbers in the aqueous phase. There was no other inoculum added and all cells originated from the bitumen itself, most likely from the small water droplets that ascended to the surface upon incubation in the microcosms (Figure 1B). During the first 150 days, the cell numbers in the planktonic phase tripled in all four microcosms from $4.3 \times 10^5 \pm 3.7 \times 10^5$ to $1.4 \times 10^6 \pm 3.7 \times 10^5$ cells mL^{-1} on average. The growth rates increased after ∼500 days, resulting in an average cell count of $1.4 \times 10^7 \pm 1.2 \times 10^6$ cells mL^{-1} after 945 days of incubation.

A community analysis based on the V3−4 region of 16S rRNA gene sequences indicated similar oil-degrading communities developed in the four microcosms over the time (Figure 2).

At the start of the incubation, the microbial communities in all four microcosms were mainly dominated by the genera *Pseudomonas* (OTU 8), *Tepidiphilus* (OTU 20), *Anaerobacillus* (OTU 44), and members of the class *Bacilli* (OTU 42) at relative abundances

of 54.9 ± 8.2%, 7.4 ± 5.0%, 4.1 ± 3.1%, and 4.4 ± 2.2% respectively. The low cell count and low number of rRNA genes after extraction of these samples agree with the assumption that the cells originate from the little water droplets enclosed in the oil sample.

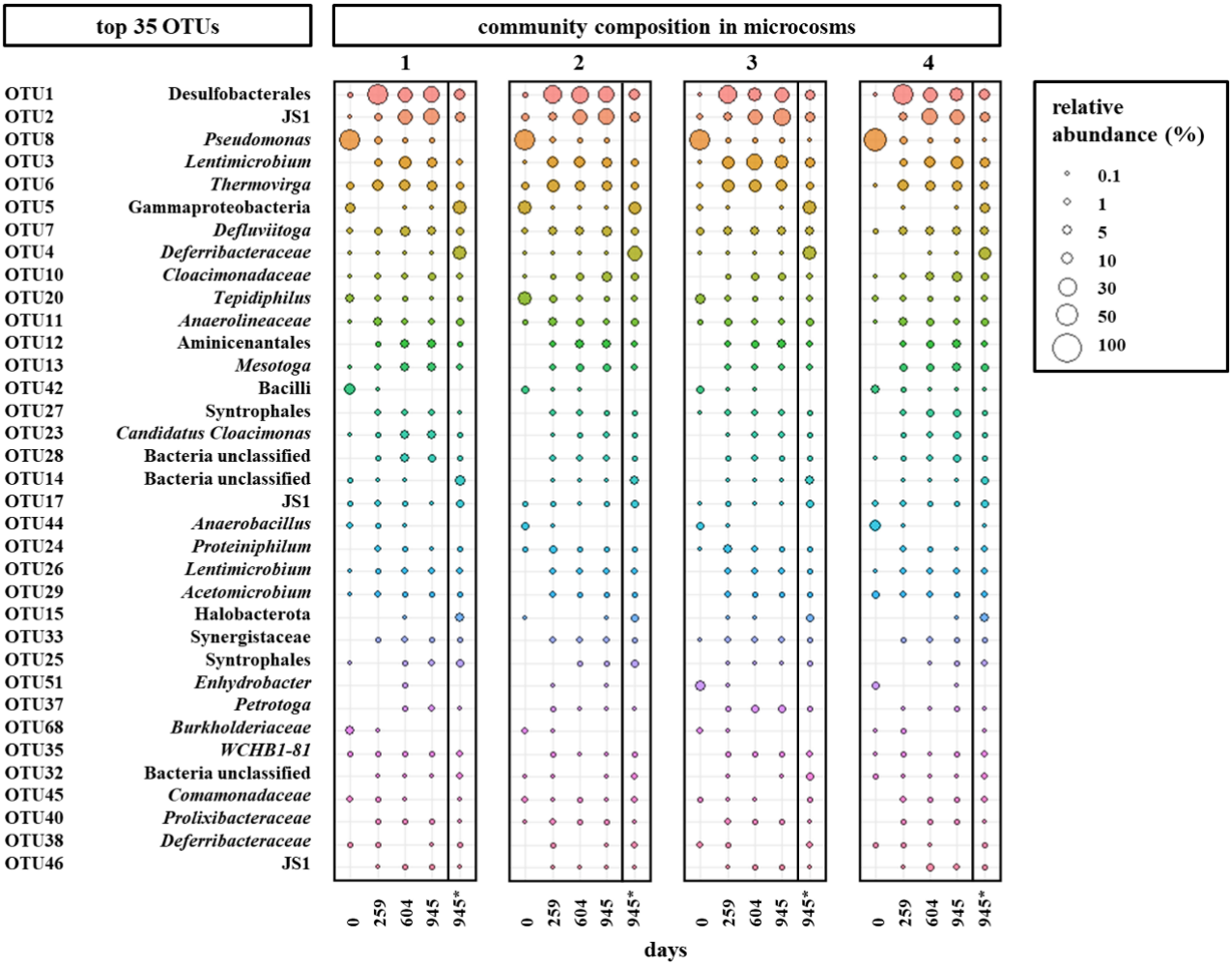


Figure 2. Microbial community compositions of the water phase and the surface biofilm of the four microcosms showing the relative abundance of the 35 most abundant operational taxonomic units (OTUs) on 97% similarity level in all four microcosms. Planktonic communities were analyzed at days 0, 259, 604, and 945 and the community of the water-bitumen interface (*) at day 945, only.

However, the four originally dominating OTUs almost disappeared after 259 days of incubation. The most abundant OTU 1 after 259 days belonged to the order *Desulfobacterales* with a relative abundance of 29.2 ± 11.5% averaged over all four microcosms indicating a succession within the microbial community which might be correlated to the availability of sulfate as electron acceptor instead of methanogenesis in the original samples. Members of the order *Desulfobacterales* are very versatile comprising strictly anaerobic sulfate-reducing,

chemoorganotrophic, chemolithoheterotrophic, or chemolithoautotrophic bacteria, and some are able to ferment.[63,71,72] Other abundant members of the community belonged to the candidate class JS1 (OTU 2), and the genera *Lentimicrobium* (OTU 3), *Thermovirga* (OTU 6), and *Defluviitoga* (OTU 7). Many prevalent community members such as representatives of *Desulfobacterales*, *Comamonadaceae*, *Synergistaceae*, *Petrotogaceae*, *Deferribacteraceae*, and *Tepidiphilus* were also earlier found to be present in Pitch Lake water droplets. However, the evolved abundances in our setups are not reflecting the natural abundances due to artificial influences such as an enlarged water volume favoring faster growing microorganisms, the dilution of toxic or growth inhibiting compounds such as selenate (sulfate analog inhibiting SRB growth) or an increased overall amount of available sulfate.[24,27] Nevertheless, representatives of these microorganisms were also found in other oil reservoirs and are known to be fermentative and sulfate-reducing oil degraders.[65,69,70]

After 945 days of incubation, the bitumen−water interface samples showed different microbial communities compared to those in the planktonic phase. Besides the abundant OTUs 1 (9.6 ± 1.4%), 2 (7.1 ± 0.9%), 3 (4.6 ± 2.4%), 6 (3.5 ± 0.9%), and 7 (4.1 ± 1.0%) that were also found in the water phase, the biofilm community was dominated by OTUs 4 and 5 belonging to the *Deferribacteraceae* and Gammaproteobacteria with an abundance of 17.9 ± 3.0% and 13.8 ± 3.7%, respectively, but the abundance of 0.04 ± 0.03% and 0.02 ± 0.02% in the water phase, respectively, was minor. The family *Deferribacteraceae* is known for anaerobic $Fe^{(III)}$, $Mn^{(IV)}$, or nitrate reduction, but also fermentation. *Deferribacteriaceae* were also found to be part of biofilms, previously.[68,73] Similarly, OTUs 14, 15, and 17 (unclassified bacteria, Halobacterota, and candidate class JS1) were mainly present at the bitumen−water interface. Nevertheless, $Fe^{(III)}$ or $Mn^{(IV)}$ oxides as electron acceptors are generally only abundant in low concentration in oil reservoirs around the globe and are not known to have a prevalent impact on the oil degradation process.[3,8]

Colonization and Biofilm Formation on Bitumen. Biofilms are a very successful form of life and make microorganisms resistant to stress, for instance protection against toxic compounds, protection against dehydration, or helping to overcome mass transfer limitations for nutrient transport and many more.[74−76] We stained pieces of the bitumen surface with the DNA stain Syto9 to visualize attached microbial cells in biofilms. Additionally, we used fluorescencelabeled lectins targeting mono- and polysaccharides which may be part of the EPS matrix (Figure 3).[74,77,78]

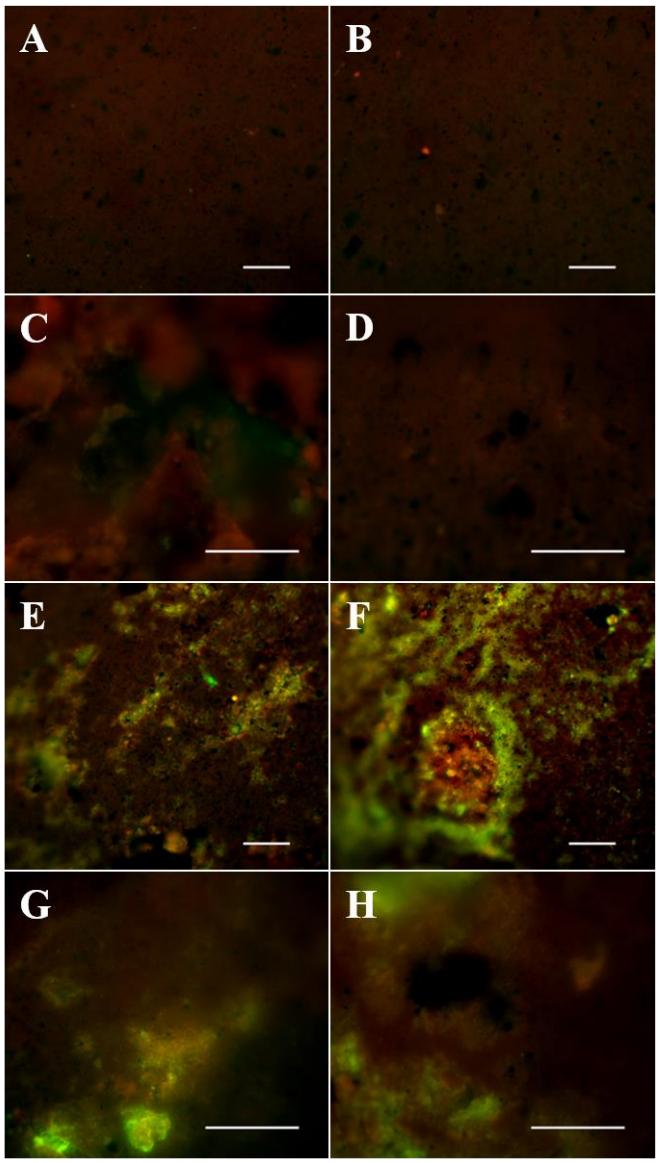


Figure 3. Fluorescence images of bitumen surfaces after 945 days of incubation in the microcosms. Images A and B show bitumen controls stained with the Syto9-lectin mix that were stored at 4 °C without incubation. Images C (microcosm 3) and D (microcosm 4) depict unstained controls of the bitumen surface in our microcosms after 945 days incubation. Images E−H show the bitumen surface of microcosms 1 (E and F), 3 (G), and 4 (H) after 945 days of incubation stained with the Syto9-lectin mix. All scale bars indicate 50 μm.

The chosen lectins covered a broad range of the most common EPS polysaccharides. Positive controls with drinking water biofilms showed the functioning of the lectin staining and no evidence for lectin−lectin binding or unspecific inhibition of the staining (Figure S1).[59,77,79] The nonincubated but stained bitumen controls (Figure 3, A and B) showed a weak fluorescence and heterogeneity of the bitumen surface. Only occasionally, strongly fluorescent dots were observed, most likely from stained single microbial cells on the surface,

but no significant fluorescence of the added lectins was visible. However, images of nonstained bitumen after 945 days incubation showed the formation of structures on the surface (Figure 3, C and D) and these structures did not appear in any nonincubated control. In contrast to unstained sample controls, stained bitumen surfaces in our microcosms after 945 days of incubation (Figure 3, E−H) showed prominent red and green fluorescent structures and aggregates, which are located in cavities and cracks along the uneven bitumen surface. The green fluorescent parts (presumably Syto9-stained cells) are mostly surrounded by a red fluorescent corona (presumably lectin-stained EPS). The weaker red signal was overlain by the green signal and is therefore less dominant in the merged pictures. Unfortunately, we were not able to take pictures at a larger magnification because of the spiky character of the surface and a strong bitumen shifting while excitation. However, the arrangement and shape of the present structures strongly indicated the formation of biofilms at the bitumen−water interface. Cell numbers were only determined from liquid samples, but the biofilms indicate that very likely a large part of the cells was attached to the bitumen surface which escaped counting in the liquid samples.

Water Content and Distribution of Droplets in the Bitumen. Seven CT scans with a total volume of 8.45 mL bitumen revealed 6330 water droplets (Figure 4). Most of these droplets were rather small with an average size of 2.18 nL. Only about 0.2% of the water droplets were larger than 0.1 μL including the three largest with 0.42, 0.55, and 2.33 μL.

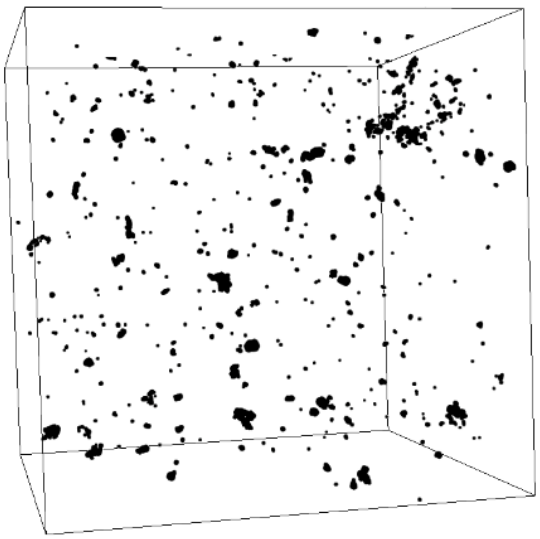


Figure 4. Partial section of a scan of a bitumen column, showing the spatial distribution of water droplets (black) in the bitumen (white). The cube's edge length is 10 mm. The largest depicted droplet here has a volume of 54 nL. The image was created with Fiji using a data set from a single CT scan.

The total droplet volume accounted for an average water content of 2.5 μL per mL bitumen with an average bitumen−water surface area of 1.13 cm^2 mL^{-1}, which is ~50-times less than the overall water content of the bitumen described by Meckenstock et al., (2014).[26] Only a minor part of the total water content was present in larger water droplets which can serve as a micro habitat. Our measurements show the size and distribution of enclosed water droplets, however it remains still unclear how fast these droplets ascent through the bitumen and if they merge or split during their way to the surface under natural conditions, since the exact temperature, pressure, and pore size in the deep subsurface reservoir remain rather unknown. Nonetheless, considering the bitumen density of 1.15 g cm^{-3} and the mean bitumen mass (104.97 g corresponds to 91.28 mL) filled into the microcosms, the overall bitumen−water surface of our microcosms was enlarged by additional 310.5% (103.1 cm^2) of the visible bitumen−water surface (33.2 cm^2).

On the basis of the assumption that the bitumen−water surface area is a limiting parameter for the overall degradation process (bioavailability of organic compounds by diffusion and dissolution), we calculated the degradation rates per surface area by using the average amount of produced CO_2 from the microcosms (1.13 mmol $year^{-1}$). However, former experiments showed that heating lowers the bitumen viscosity and enhanced the buoyance density-driven up-rise of water droplets toward the bitumen surface within days. Therefore, it is likely that most droplets reached the overlaying water phase within a few days after the setup.[26,27] Hence, we calculated the degradation rate per bitumen-water surface area either based on the visible surface area only (33.2 cm^2) or based on the visible surface area plus the assumed dropletwater surface area (136.3 cm^2) in our microcosms, resulting in 8.3−34.1 μmol CO_2 cm^{-2} $year^{-1}$, respectively.

We then estimated the theoretical degradation rate in a regular bitumen sample based on the biodegradation rates per surface area and the assumption that the degradation would only take place in the water droplets dispersed in the bitumen. Considering the water droplet surface area per volume of bitumen (1.13 cm^2 mL^{-1}) led to a degradation rate of 9.4−38.6 mmol CO_2 L^{-1} bitumen $year^{-1}$. Assuming an average chemical formula of the hydrocarbons in the bitumen of $C_xH_{1.4x}$ ($M_w \approx 13.5$ g mol^{-1}), the CO_2-production accounts for a biodegradation of 0.13−0.52 g carbon L^{-1} bitumen $year^{-1}$. However, the relatively big volume of the overlaying water added to the microcosms might have affected the microbial activity to a certain degree. Furthermore, the cohesive oil−water transition zone and the available sulfate amount artificially increased the degradation rates in our experimental microcosms and are probably leading to an overestimation of the degradation rates. Nonetheless, the degradation

rates resulted in decay times of around 2200−9000 years for a liter of bitumen, which is indeed on a geological time scale, although it is still up to 15-times higher compared to methanogenesis as final electron-accepting process in crude oil.[64,80] We conclude that the overall bioavailable oil−water interface area is notably enlarged by dispensed water droplets in oil. Our results suggest that this facet of microbial life in oil reservoirs has the potential to substantially contribute to the overall biodegradation of oil over millions of years in addition to degradation at the oil−water-transition-zone. Furthermore, we could show that the reverse stable isotope measurements allowed to assess such slow biodegradation rates in the range of geological time scales.

ASSOCIATED CONTENT

*Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c00596.

Details about geochemical measurements, DNA extraction, biofilm visualization, sulfate determination, 3D-imaging of bitumen, and additional tables and figures (PDF)

AUTHOR INFORMATION

Corresponding Author

Rainer U. Meckenstock − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*; orcid.org/0000-0001-7786-9546; Phone: +49 (0201) 183−6601; Email: rainer.meckenstock@uni-due.de

Authors

Mark Pannekens − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*; orcid.org/0000-0003-3200-0390

Lisa Voskuhl − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

Sadjad Mohammadian − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

Daniel Köster − *Instrumental Analytical Chemistry, University of Duisburg-Essen, 45141 Essen, Germany*

Arne Meier − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

John M. Köhne − *Department of Soil System Science, Helmholtz Centre for Environmental Research, 06120 Halle, Germany*

Michelle Kulbatzki − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

Ali Akbari − *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

Shirin Haque − *Department of Physics, Faculty of Science and Technology, The University of The West Indies, St. Augustine, Trinidad and Tobago*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.1c00596

Funding

This work was supported by the ERC advanced grant EcOILogy [grant number 666952] funded by the European Research Council and the German Research Foundation [grant number BR 5493/1−1], and the EU Horizon 2020 project Reground [grant number 641768].

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Christian Thyssen for his support with sample preparation for ion chromatography. Till Bornemann supported with advice for the cleaning steps during DNA extraction. We thank Riad Hossein for assisting the sampling campaigns in Trinidad.

4.3.4 REFERENCES

(1) Head, I. M.; Jones, D. M.; Larter, S. R. Biological activity in the deep subsurface and the origin of heavy oil. *Nature* 2003, 426, 344.

(2) Aitken, C. M.; Jones, D. M.; Larter, S. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature* 2004, 431,291−294.

(3) Youssef, N.; Elshahed, M. S.; McInerney, M. J. Microbial processes in oil fields: culprits, problems, and opportunities. *Adv. Appl. Microbiol*. 2009, 66, 141−251.

(4) Kayukova, G.; Uspensky, B.; Abdrafikova, I.; Musin, R. Characteristic features of the hydrocarbon composition of Spiridonovskoe (Tatarstan) and Pitch Lake (Trinidad and Tobago) asphaltites. *Pet. Chem*. 2016, 56, 572−579.

(5) Mbadinga, S. M.; Wang, L.-Y.; Zhou, L.; Liu, J.-F.; Gu, J.-D.; Mu, B.-Z. Microbial communities involved in anaerobic degradation of alkanes. *Int. Biodeterior. Biodegrad*. 2011, 65, 1−13.

(6) Larter, S.; Wilhelms, A.; Head, I.; Koopmans, M.; Aplin, A.; Di Primio, R.; Zwach, C.; Erdmann, M.; Telnaes, N. The controls on the composition of biodegraded oils in the deep subsurface - part 1: biodegradation rates in petroleum reservoirs. *Org. Geochem*. 2003, 34, 601−613.

(7) Widdel, F.; Rabus, R. Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr. Opin. Biotechnol*. 2001, 12, 259−276.

(8) Lovley, D. R.; Chapelle, F. H. Deep subsurface microbial processes. Rev. *Geophys*. 1995, 33, 365−381.

(9) Pannekens, M.; Kroll, L.; Müller, H.; Mbow, F. T.; Meckenstock, R. U. Oil reservoirs, an exceptional habitat for microorganisms. *New Biotechnol*. 2019, 49, 1−9.

(10) Li, X.-X.; Mbadinga, S. M.; Liu, J.-F.; Zhou, L.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z. Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. *Int. Biodeterior. Biodegrad*. 2017, 120, 170−185.

(11) Meckenstock, R. U.; Elsner, M.; Griebler, C.; Lueders, T.; Stumpp, C.; Aamand, J.; Agathos, S. N.; Albrechtsen, H.-J.; Bastiaens, L.; Bjerg, P. L.; Boon, N.; Dejonghe, W.; Huang, W. E.; Schmidt, S. I.; Smolders, E.; Sørensen, S. R.; Springael, D.; van Breukelen, B. M. Biodegradation: updating the concepts of control for microbial cleanup in contaminated aquifers. *Environ. Sci. Technol*. 2015, 49, 7073−7081.

(12) Jiménez, N.; Richnow, H. H.; Vogt, C.; Treude, T.; Krüger, M. Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *J. Mol. Microbiol Biotechnol* 2016, 26, 227−242.

(13) Gieg, L. M.; Davidova, I. A.; Duncan, K. E.; Suflita, J. M. Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. *Environ. Microbiol*. 2010, 12, 3074−3086.

(14) Gieg, L. M.; Fowler, S. J.; Berdugo-Clavijo, C. Syntrophic biodegradation of hydrocarbon contaminants. *Curr. Opin. Biotechnol*. 2014, 27, 21−29.

(15) Jones, D. M.; Head, I. M.; Gray, N. D.; Adams, J. J.; Rowan, A. K.; Aitken, C. M.; Bennett, B.; Huang, H.; Brown, A.; Bowler, B. F. J.; Oldenburg, T.; Erdmann, M.; Larter, S.

R. Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* 2008, 451, 176−180.

(16) Xu, D.; Zhang, K.; Li, B.-G.; Mbadinga, S. M.; Zhou, L.; Liu, J.- F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z. Simulation of *in situ* oil reservoir conditions in a laboratory bioreactor testing for methanogenic conversion of crude oil and analysis of the microbial community. *Int. Biodeterior. Biodegrad*. 2019, 136, 24−33.

(17) Head, I.; Larter, S.; Gray, N.; Sherry, A.; Adams, J.; Aitken, C.; Jones, D.; Rowan, A.; Huang, H.; Röling, W. Hydrocarbon degradation in petroleum reservoirs. *In Handbook of Hydrocarbon and Lipid Microbiology*; Springer: New York, 2010; pp 3097−3109.

(18) Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F. Anaerobic bacterial metabolism of hydrocarbons. *FEMS microbiology reviews* 1998, 22, 459−473.

(19) Meckenstock, R. U.; Boll, M.; Mouttaki, H.; Koelschbach, J. S.; Tarouco, P. C.; Weyrauch, P.; Dong, X.; Himmelberg, A. M. Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. *J. Mol. Microbiol Biotechnol* 2016, 26, 92−118.

(20) Aeckersberg, F.; Bak, F.; Widdel, F. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch. Microbiol*. 1991, 156, 5−14.

(21) Schulte, S.; Köster, D.; Jochmann, M.; Meckenstock, R. Applying reverse stable isotope labeling analysis by mid-infrared laser spectroscopy to monitor BDOC in recycled wastewater. *Sci. Total Environ*. 2019, 665, 1064−1072.

(22) Dong, X.; Jochmann, M. A.; Elsner, M.; Meyer, A. H.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U. Monitoring microbial mineralization using reverse stable isotope labeling analysis by mid-infrared laser spectroscopy. *Environ. Sci. Technol.* 2017, 51, 11876−11883.

(23) Dong, X.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U. Quantification of microbial degradation activities in biological activated carbon filters by reverse stable isotope labelling. *AMB Express* 2019, 9, 109.

(24) Stoeva, M. K.; Coates, J. D. Specific inhibitors of respiratory sulfate reduction: towards a mechanistic understanding. *Microbiology* 2019, 165, 254−269.

(25) Bennett, B.; Adams, J.; Gray, N.; Sherry, A.; Oldenburg, T.; Huang, H.; Larter, S.; Head, I. 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.* 2013, 56, 94−105.

(26) Meckenstock, R. U.; von Netzer, F.; Stumpp, C.; Lueders, T.; Himmelberg, A. M.; Hertkorn, N.; Schmitt-Kopplin, P.; Harir, M.; Hosein, R.; Haque, S.; Schulze-Makuch, D. Water droplets in oil are microhabitats for microbial life. *Science* 2014, 345, 673−676.

(27) Pannekens, M.; Voskuhl, L.; Meier, A.; Müller, H.; Haque, S.; Frösler, J.; Brauer, V.; Meckenstock, R. Densely populated water droplets in heavy oil seeps. *Appl. Environ. Microbiol.* 2020, 86, 1−12.

(28) Kim, J. S.; Crowley, D. E. Microbial diversity in natural asphalts of the rancho la brea tar pits. *Appl. Environ. Microbiol*. 2007, 73, 4579−4591.

(29) Ait-Langomazino, N.; Sellier, R.; Jouquet, G.; Trescinski, M. Microbial degradation of bitumen. *Experientia* 1991, 47, 533−539.

(30) Schneiker, S.; dos Santos, V. A. P. M.; Bartels, D.; Bekel, T.; Brecht, M.; Buhrmester, J.; Chernikova, T. N.; Denaro, R.; Ferrer, M.; Gertler, C.; Goesmann, A.; Golyshina, O. V.; Kaminski, F.; Khachane, A. N.; Lang, S.; Linke, B.; McHardy, A. C.; Meyer, F.; Nechitaylo, T.; Puhler, A.; Regenhardt, D.; Rupp, O.; Sabirova, J. S.; Selbitschka, W.; Yakimov, M. M.; Timmis, K. N.; Vorholter, F. J.; Weidner, S.; Kaiser, O.; Golyshin, P. N. Genome sequence of the ubiquitous hydrocarbondegrading marine bacterium *Alcanivorax borkumensis*. *Nat. Biotechnol*. 2006, 24, 997−1004.

(31) Golby, S.; Ceri, H.; Gieg, L. M.; Chatterjee, I.; Marques, L. L.; Turner, R. J. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. *FEMS Microbiol. Ecol*. 2012, 79, 240−250.

(32) Wolf, M.; Bachofen, R. Microbial legradation of bitumen. *Experientia* 1991, 47, 542−548.

(33) Widdel, F.; Pfennig, N. Studies on dissimilatory sulfatereducing bacteria that decompose fatty acids. *Arch. Microbiol*. 1981, 129, 395−400.

(34) Babu, D. R.; Cormack, D. E. Effect of low-temperature oxidation on the composition of Athabasca bitumen. *Fuel* 1984, 63, 858−861.

(35) Kapadia, P. R.; Kallos, M. S.; Gates, I. D. A review of pyrolysis, aquathermolysis, and oxidation of Athabasca bitumen. *Fuel Process. Technol*. 2015, 131, 270−289.

(36) Jia, N.; Moore, R.; Mehta, S.; Van Fraassen, K.; Ursenbach, M.; Zalewski, E. In Compositional changes for Athabasca bitumen in the presence of oxygen under low temperature conditions, *Canadian International Petroleum Conference*, 2004; Petroleum Society of Canada: 2004.

(37) Strausz, O. P.; Jha, K. N.; Montgomery, D. S. Chemical composition of gases in Athabasca bitumen and in low-temperature thermolysis of oil sand, asphaltene and maltene. *Fuel* 1977, 56, 114−120.

(38) Merdrignac, I.; Espinat, D. Physicochemical characterization of petroleum fractions: the state of the art. *Oil Gas Sci. Technol*. 2007, 62, 7−32.

(39) Selucky, M. L.; Chu, Y.; Ruo, T.; Strausz, O. P. Chemical composition of Athabasca bitumen. *Fuel* 1977, 56, 369−381.

(40) Mehrotra, A. K.; Svrcek, W. Y. Viscosity of compressed Athabasca bitumen. *Can. J. Chem. Eng*. 1986, 64, 844−847.

(41) Badamchi-Zadeh, A.; Yarranton, H.; Svrcek, W.; Maini, B. Phase behaviour and physical property measurements for VAPEX solvents: Part I. Propane and Athabasca bitumen. J. *Can. Pet Technol*. 2009, 48, 54−61.

(42) Del Bianco, A. Composition and analysis of heavy petroleum *fractionsAppl. Catal*., A. 1994; Vol. 114.N29N30.

(43) Mckenna, A. M.; Purcell, J. M.; Rodgers, R. P.; Marshall, A. G. Heavy petroleum composition. 1. Exhaustive compositional analysis of Athabasca bitumen HVGO distillates by Fourier transform ion cyclotron resonance mass spectrometry: A definitive test of the Boduszynski model. Energy *Fuels* 2010, 24, 2929−2938.

(44) Hayes, M.; Stacey, M.; Standley, J.; Entwistle, A. Studies on bitumen: Part 3. Experiments on the biodegradation of bitumen by soil micro-organisms. *Fuel* 1972, 51, 146−149.

(45) Phillips, D.; Hitchon, J.; Johnson, D.; Matthews, J. The radiation swelling of bitumens and bitumenised wastes. J. *Nucl. Mater*. 1984, 125, 202−218.

(46) Gehringer, P.; Proksch, E.; Szinovatz, W. The gamma radiolysis of bitumen. Pt. 1. *Atomkernenergie Kerntechnik* 1981, 39, 12−15.

(47) Mello, M.; Braz, D.; Motta, L.; Leite, L. Investigation on the effects of gamma irradiation on bitumen. 2011.

(48) Mouazen, M.; Poulesquen, A.; Bart, F.; Masson, J.; Charlot, M.; Vergnes, B. Rheological, structural and chemical evolution of bitumen under gamma irradiation. *Fuel Process. Technol*. 2013, 114, 144−153.

(49) Christman, G. D.; León-Zayas, R. I.; Zhao, R.; Summers, Z. M.; Biddle, J. F. Novel clostridial lineages recovered from metagenomes of a hot oil reservoir. *Sci. Rep.* 2020, 10, 1−11.

(50) Da Cunha, C. D.; Rosado, A. S.; Sebastián, G. V.; Seldin, L.; Von der Weid, I. Oil biodegradation by Bacillus strains isolated from the rock of an oil reservoir located in a deep-water production basin in Brazil. *Appl. Microbiol. Biotechnol*. 2006, 73, 949−959.
(51) Rosnes, J. T.; Torsvik, T.; Lien, T. Spore-forming thermophilic sulfate-reducing bacteria isolated from North Sea oil field waters. *Appl. Environ. Microbiol.* 1991, 57, 2302−2307.
(52) Assayag, N.; Rivé, K.; Ader, M.; Jézéquel, D.; Agrinier, P. Improved method for isotopic and quantitative analysis of dissolved inorganic carbon in natural water samples. Rapid Commun. *Mass Spectrom*. 2006, 20, 2243−2251.
(53) van Geldern, R.; Nowak, M. E.; Zimmer, M.; Szizybalski, A.; Myrttinen, A.; Barth, J. A.; Jost, H.-J. R. Field-based stable isotope analysis of carbon dioxide by mid-infrared laser spectroscopy for carbon capture and storage monitoring. *Anal. Chem*. 2014, 86, 12191−12198.
(54) Coplen, T. B. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Commun. *Mass Spectrom*. 2011, 25, 2538−2560.
(55) Köster, D.; Wolbert, J.-B.; Schulte, M. S.; Jochmann, M. A.; Schmidt, T. C. Origin of xylitol in chewing gum: A compound-specific isotope technique for the differentiation of corn-and wood-based xylitol by LC-IRMS. *J. Agric. Food Chem*. 2018, 66, 2015−2020.
(56) Himmelberg, A. *Life in Pitch-Bacteria in a Natural Oil Emitting Lake Help to Understand Anaerobic Biodegradation of Polycyclic Aromatic Hydrocarbons*, PhD-Thesis; Technical University of Munich, 2019.
(57) Tebbe, C. C.; Vahjen, W. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microb*iol. 1993, 59, 2657−2665.
(58) Thyssen, C. *Biofilm Formation by the Manganese-Oxidizing Bacterium Leptothrix discophora Strain SS-1 and Corrosion of Stainless Steel, PhD-thesis*; University of Duisburg-Essen, 2018.
(59) Strathmann, M.; Wingender, J.; Flemming, H.-C. Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*. *J. Microbiol. Methods* 2002, 50, 237−248.
(60) Shiina, N.; Tateno, H.; Ogawa, T.; Muramoto, K.; Saneyoshi, M.; Kamiya, H. Isolation and characterization of L-rhamnose-binding lectins from chum salmon (Oncorhynchus keta) eggs. *Fish. Sci*. 2002, 68, 1352−1366.
(61) Neu, T. R.; Swerhone, G. D.; Lawrence, J. R. Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. *Microbiology* 2001, 147, 299−313.

(62) Muyzer, G.; Stams, A. J. The ecology and biotechnology of sulphate-reducing bacteria. *Nat. Rev. Microbiol*. 2008, 6, 441−454.

(63) Kuever, J.; Rainey, F. A.; Widdel, F. Desulfobacterales ord. nov. *Bergey's Manual of Systematics of Archaea and Bacteria* 2015, 1−7.

(64) Gieg, L. M.; Duncan, K. E.; Suflita, J. M. Bioenergy production via microbial conversion of residual oil to natural gas. *Appl. Environ. Microbiol.* 2008, 74, 3022−3029.

(65) Ollivier, B.; Borgomano, J.; Oger, P. Petroleum: from formation to microbiology. *Microbial Life of the Deep Biosphere* 2014, 8, 161−185.

(66) Zhou, Z.; Liang, B.; Wang, L.-Y.; Liu, J.-F.; Mu, B.-Z.; Shim, H.; Gu, J.-D. Identifying the core bacterial microbiome of hydrocarbon degradation and a shift of dominant methanogenesis pathways in the oil and aqueous phases of petroleum reservoirs of different temperatures from China. *Biogeosciences* 2019, 16, 4229−4241.

(67) Toth, C. R.; Gieg, L. M. Time course-dependent methanogenic crude oil biodegradation: dynamics of fumarate addition metabolites, biodegradative genes, and microbial community composition. *Front. Microbiol.* 2018, 8, 2610.

(68) Huber, H.; Stetter, K. O. Deferribacteraceae fam. nov. *Bergey's Manual of Systematics of Archaea and Bacteria* 2015, 1−1.

(69) Dahle, H.; Birkeland, N.-K. *Thermovirga lienii gen. nov., sp. nov*., a novel moderately thermophilic, anaerobic, amino-acid-degrading bacterium isolated from a North Sea oil well. Int. J. Syst. *Evol. Microbiol*. 2006, 56, 1539−1545.

(70) Sun, L.; Toyonaga, M.; Ohashi, A.; Tourlousse, D. M.; Matsuura, N.; Meng, X.-Y.; Tamaki, H.; Hanada, S.; Cruz, R.; Yamaguchi, T.; Sekiguchi, Y. *Lentimicrobium saccharophilum gen. nov., sp. nov.*, a strictly anaerobic bacterium representing a new family in the phylum Bacteroidetes, and proposal of *Lentimicrobiaceae fam. nov*. *Int. J. Syst. Evol. Microbiol.* 2016, 66, 2635−2642.

(71) Kuever, J., The Family *Desulfobulbaceae*. In The Prokaryotes; Springer: New York, 2014; pp 75−86.

(72) Zapata-Peñasco, I.; Salazar-Coria, L.; Saucedo-García, M.; Villa-Tanaka, L.; Hernández-Rodríguez, C. Bisulfite reductase and nitrogenase genes retrieved from biocorrosive bacteria in saline produced waters of offshore oil recovery facilities. *Int. Biodeterior. Biodegrad*. 2013, 81, 17−27.

(73) Daghio, M.; Gandolfi, I.; Bestetti, G.; Franzetti, A.; Guerrini, E.; Cristiani, P. Anodic and cathodic microbial communities in single chamber microbial fuel cells. *New Biotechnol.* 2015, 32, 79−84.

(74) Flemming, H.-C. The perfect slime. Colloids Surf., B 2011, 86, 251−259.

(75) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2004, 2, 95−108.

(76) Johnsen, A.; Karlson, U. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. *Appl. Microbiol. Biotechnol*. 2004, 63, 452−459.

(77) Sutherland, I. W. Polysaccharases for microbial exopolysaccharides. *Carbohydr. Polym*. 1999, 38, 319−328.

(78) Flemming, H. C.; Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol.* 2010, 8, 623−33.

(79) Czaczyk, K.; Myszka, K. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Pol J. Environ. Stud 2007*, 16, 799−806.

(80) Berdugo-Clavijo, C.; Gieg, L. M. Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production waters. *Front. Microbiol*. 2014, 5, 197.

4.3.5 Supporting Information - Microbial degradation rates of natural bitumen

*Mark Pannekens[1], Lisa Voskuhl[1], Sadjad Mohammadian[1], Daniel Köster[2], Arne Meier[1], John. M. Köhne[3], Michelle Kulbatzki[1], Ali Akbari[1], Shirin Haque[4], Rainer U. Meckenstock[1]**

[1] *Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany*

[2] *Instrumental Analytical Chemistry, University of Duisburg-Essen, 45141 Essen, Germany*

[3] *Department of Soil System Science, Helmholtz Centre for Environmental Research, 06120 Halle, Germany*

[4] *Department of Physics, Faculty of Science and Technology, The University of The West Indies, St. Augustine, Trinidad and Tobago*

* Corresponding Author: Rainer U. Meckenstock, Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, 45141 Essen, Germany, rainer.meckenstock@uni-due.de, Tel.: +49 (0201) 183 - 6601

Number of pages: 13

Number tables: 3

Number of figures: 1

List of content

Experimental section and results

Experimental section and results

Geochemistry of water extracted from the bitumen body

For anion analysis, water samples extracted from bitumen were diluted with 1 M KOH (1:10) and centrifuged for 15 min at 16,000 x g. The supernatant was further diluted in deionized water (18.2 MΩ, TOC > 5ppb; 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) 1:10 and 1:100, respectively. For cation analysis, water samples were diluted 1:10 with deionized water and centrifuged for 15 min at 16,000 x g. The supernatant was further diluted with deionized water 1:10. Cations were measured with a 850 professional ion chromatography system (Metrohm, Germany) equipped with a Metrosep C4 S-Guard/4.0 guard column and Metrosep C4 250/4.0 analytical column. The eluent consisted of 1.7 mM nitric acid (Carl Roth, Germany) and 0.7 mM dipicolinic acid (Fluka™, UK). The flow rate was 0.9 ml min^{-1} at room temperature. The software Magic IC™ Net (Metrohm, Germany) version 2.3 was used for data analysis. Anions were measured with a Dionex DX 500 AS3500 ion chromatography system (Dionex, CA, USA) equipped with a ASRD Ultra II suppressor, a CD20 conductivity detector, a EG 50 gradient generator combined with an EluGen cartridge EGC II KOH, a AG17C 2x50 mm guard column and a AS17C 2x250 mm analytical column. A KOH gradient was applied starting with 10 mM for 1 min following a linear increase over 4.5 min to 50 mM. Afterwards, the concentrations declined over 1 min to 10 mM and remained constant for an additional minute before the next measurement. The flowrate was 0.38 ml min^{-1}. The software Chromeleon version 6.70 Build 1820 was used for data analysis. The results are shown in table S1.

Table S1. Measured ion concentrations (± standard deviation) in water extracted from Pitch Lake bitumen.

anion	**concentration [mM]**	**cation**	**concentration [mM]**
chloride	433.0 ± 27.0	sodium	218.4 ± 12.7
nitrite	0.0	ammonium	16.4 ± 0.6
sulfate	9.7 ± 3.9	potassium	5.5 ± 0.4
nitrate	0.0	calcium	1.1 ± 0.1
phosphate	31.7 ± 4.4	magnesium	1.2 ± 0.2

Droplet origin

A direct comparison of extracted water with seawater sampled from the coast nearby and surface water sampled from ponds on the Pitch Lake (presumably rainwater) showed strong differences in ion concentration and composition (results not shown), which agrees with Meckenstock et al., 2014, which compared the isotopic ratios of oxygen and hydrogen of extracted water with the local meteoric water line, indicating an origin from the deep subsurface.[1] Further, our 16S gene sequencing results show a consistently anaerobic community with nearly no aerobic surface contaminations (figure 2). These findings together strongly support a deep subsurface origin of our entrapped water droplets present in the Pitch Lake bitumen.

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

For DNA extraction of sessile cells, the remaining water was removed from the microcosms. The microcosms were opened and subsequently stored at -70 °C for 4 h. Scalpels were used to flake off thin pieces of the frozen bitumen surface. These pieces were collected with tweezers and transferred into bead-beating tubes containing 0.1 mm glass beads, ranging from 0.2 g to 0.7 g. Afterwards, 300 µl Miller phosphate buffer (Carl ROTH GmbH + Co. KG), 300 µl Miller SDS lysis buffer (Bernd Kraft GmbH, Thermo Fisher Scientific), and 600 µl phenol-chloroform-isoamyl alcohol solution [PCI, 25:24:1 (vol:vol:vol:), Thermo Fisher Scientific, USA] were added and gently mixed. Cell lysis was performed by bead-beating for 45 s at 5.5 m s^{-1}. Afterwards samples were centrifuged at 10.000 x g at 4 °C for 5 min. The aqueous supernatant was transferred to a phase lock gel heavy tube (Eppendorf, Germany), followed by the addition of an equal amount of chloroform-isoamyl alcohol solution [CI, 24:1 (vol:vol), Thermo Fisher Scientific, USA] and gentle mixing. Subsequently, the samples were centrifuged again at 10,000 x g at 4 °C for 5 min, and the supernatant of the upper layer was transferred into a new 2 ml reaction tubes. The samples were further proceeded by using the DNeasy PowerLyser Power Soil Kit (Qiagen GmbH, Germany) for DNA extraction according to the manufacturer's instructions. As the only deviation from the standard protocol, the amount of C4 buffer at step 12 was enhanced from 1200 to 1500 µl.[2-4]

For DNA extraction of planktonic cells, 1.5 ml liquid sample (time points 0 and 4) and 0.5 ml (time points 6 and 8) were used, respectively. The DNeasy PowerLyser Power Soil Kit (Qiagen GmbH, Germany) was used for DNA extraction according to the manufacturer's instructions. Modifications of the "Quick-Start Protocol" were made as follows: in step 3. samples were incubated for 5 min at 50 °C and 300 rpm in a thermoshaker, in step 4. samples were shaken for 45 s and 4000 rpm in a Precellys 24 tissue homogenizer (Bertin technologies

SAS, France), in step 9. 500 µl supernatant was used instead of 600 µl, and in step 12. 650 µl supernatant was used instead of 750 µl.

After extraction, we used a modified purification protocol to remove remaining contaminations from the liquid sample extracts.[5-7] To this end, a solution containing 24.7 mM glycogen and 3 M sodium acetate was added (10 % v/v) to the DNA extract. Then, 400 µl of 99.5 % ethanol were added and incubated at 4 °C, overnight. Afterwards, samples were centrifuged at 15,000 x g for 30 min and the supernatant discarded. Thereafter, 200 µl 70 % ethanol were added and the tube was gently shaken. The samples were centrifuged again at 15,000 x g for 30 min and the supernatant was discarded. The samples were dried for 15 min at air and the extracted DNA was resuspended in 20 µl of C6 buffer solution of the extraction kit.

Former experiments (results not shown) showed an inhibition during the amplicon PCR of samples extracted from bitumen due to matrix effects. Therefore, a further cleaning procedure modified from the Illumina protocol was applied before amplicon PCR. The samples were centrifuged at 7,500 x g for 3 min. Afterwards, the supernatants of each triplicate were pooled. Then, 60 µl of each sample were mixed with 50 µl MagSi-NGS Prep-Plus magnetic beads (Steinbrenner Laborsysteme GmbH, Mannheim, Germany) and incubated for 5 min. After the incubation, the samples were placed on a magnetic stand for 2 min and the supernatant was discarded. The samples remained on the magnetic stand and 200 µl of 80 % ethanol were added. After 30 s incubation, the ethanol was discarded followed by 5 min drying. Then, the magnetic stand was removed and 40 µl EB buffer were added and mixed 15x by pipette followed by a 2 min incubation. Afterwards, the samples were placed on a magnetic stand for 2 min, after which the supernatant was transferred into clean tubes for further analysis.

Amplification of the 16S rRNA genes, library preparation and sequencing were performed as described in Pannekens et al., 2020 with minor modifications: The 16S rRNA gene sequences

were amplified by targeting the hypervariable V3-V4 region with forward primer S-D-Arch-0519-a-S-15 (5′-CAGCMGCCGCGGTAA-3′) and an 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 S-D-Bact-0785-b-A-18 (5′-GACTACHVGGGTATCTAATCC-3′) and an overhang adaptor (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CNV GGG TAT CTA ATC C-3′).[8] The choice of primers and hypervariable region aimed at covering the broadest possible spectrum of both bacteria and archaea.[9] The mothur version (version 1.44.0, last updated 19 March, 2019) was used for bioinformatic analysis. In addition, ambiguous bases shorter than 287 bp or longer than 289 bp were removed from the data set after merging forward and reverse reads. Version 138 of the SILVA database was used for alignment and the reads were rarified to the lowest detected read number of 8479 (sample 4_T0_1). Raw sequencing reads were deposited in the NCBI database under BioProject PRJNA645080.

Biofilm visualization on bitumen surface

The frozen bitumen surface (-70° C) was chipped off by scalpel and transferred with tweezers face up onto concaved object slides. Seven fluorescence labeled lectins with specific bounding to different polysaccharides were used for EPS visualization (table S2). A lectin solution was prepared containing Syto9 as DNA stain and 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, 0.1 mM $CaCl_2$ x $2H_2O$, 0.1 mM $MnCl_2$ x $4H_2O$, 0.1 mM $MgCl_2$ x $6H_2O$, 10 µM Sytgo9, 10 µg ml^{-1} of each lectin, and a pH of 7.5 (modified from manufacturers buffer solutions). The bitumen surface was covered with 50 µl of the lectin solution and incubated for 30 min in the dark. Afterwards, the lectin solution was removed with a pipette and the bitumen surface was gently washed three times with deionized water.[10, 11] In addition, the staining solution was tested on drinking water biofilms grown on

EPDM coupons (ethylene propylene diene monomer) according to Mackowiak (2019) (figure S1).[12] An epifluorescence microscope Eclipse Ni-E (Nikon; Japan) with FITC and DsRed filters, a CFI Plan Fluor 40x/0.75 or DLL Plan Fluor 20x/0.5 objective and the software Nis Elements version 5.11 was used for analysis of bitumen samples. A confocal laser scanning microscope and the LAS.X software version 3.7.2.22383 were used for drinking water biofilm samples according to Pannekens *et al.,* (2020).[8]

Table S2. List of used lectins, their shortcut, the specific binding and the excitation and emission wavelength of the associated fluorescent tags.

Name	**Shortcut**	**Specific binding**	**Excitation in nm**	**Emission in nm**
Concanavalin A	Con A	Mannose; Glucose; Alginate [11, 13, 14]	593	614
Peanut agglutinin	PNA	Galactose [13]	596	615
Ulex Europaeus Agglutinin I	UEA	Fucose [10, 11, 13]	652	672
Griffonia Simplicifolia Lectin II	GS II	*N-acetylglucosamine* [13]	596	615
Wheat germ agglutinin	WGA	N-acetylglucosamine [13, 14]	593	614
Soybean agglutinin	SBA	N-acetylgalactosamine; Galactose [13]	596	615
Oncorhynchus keta Lectin	CSL 3	Rhamnose [15]	554	568

Syto9-lectin tests on drinking water biofilms

To prove the staining potential and exclude the possibility of inhibiting or quenching effects due to lectin mixing, drinking water biofilms, grown for 14 days on an EPDM coupons, were stained as described above and examined with an epifluorescence microscope (figure S1).

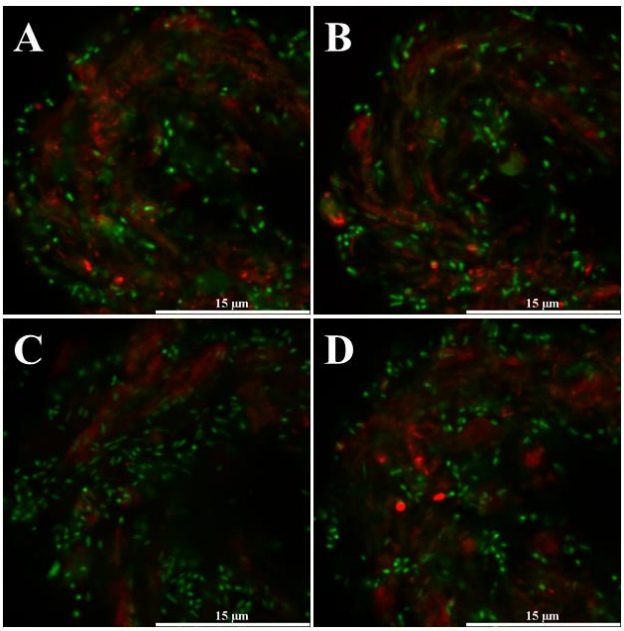


Figure S1. Fluorescence images of drinking water biofilms grown on EPDM coupons and stained with Syto9 (green) and seven different lectins (red). Pictures were taken with a confocal laser scanning microscope. Excitation wavelengths were λ 488 nm, λ 561 nm, and λ 633 nm, respectively. Scale bars indicate 15 µm.

The images clearly show green fluorescent microbial cells embedded in a red fluorescent EPS matrix. No inhibition effects were observable.

Sulfate measurements during the experiment

Liquid sample were centrifuged for 10 min at 16,000 x g, the supernatant was diluted 1:10 with 0.1 M KOH and centrifuged again for 10 min at 16,000 x g. The supernatant was then diluted 1:50 or 1:100 with deionized water (18.2 MΩ, TOC > 5ppb), respectively. Sulfate was measured by a Dionex Aquion ion chromatography system (Thermo Scientific, USA) equipped with a Dionex IonPac™ AG23-4 µm 2x50 mm guard column, a Dionex IonPac™ AG23-4 µm 2x250 mm analytical column, an AERS 500 Carbonate 2 mm suppressor, and a DS6 heated conductivity cell detector. A carbonate buffer (0.0008 M $NaHCO_3$; 0.0045 M Na_2CO_3) served as eluent, with a flow rate of 0.25 ml min^{-1} at 30 °C. The software Chromeleon (Thermo Scientific, MA, USA) version 7.2 SR5 was used for data analysis.

3D imaging of bitumen – droplet distribution

The water droplet distribution in the bitumen was determined via computed tomography scanning. Liquid oil seeping to the surface at different locations on the Pitch Lake was either filled directly on site (3 columns) or in the laboratory (3 columns) into round plastic columns (15 cm in length with a diameter of 1.5 cm or 1 cm, respectively). The top and bottom were sealed with rubber stoppers in the field and exchanged with hot glue in the laboratory. The sealed columns were placed on plastic stands and stored at 4 °C until analysis, where the heavy oil remained solid. The samples were scanned with X-ray tomography (X-tek XT H 225, Nikon Metrology, Japan) at an energy of 90 kV and a beam current of 155 µA. In total, 2000 projections were taken with 2 frames per projection and an exposure time of 1 s. The projections were then reconstructed into a 3D image using the software X-tec CT Pro 3D. The final images had 8-bit grey scale resolutions and 12.5 µm spatial resolution. The quantitative image analysis was carried out using the Fiji software (version 1.52i).[16] A rectangular inner

sub volume with a size of 880 x 880 x 1000 voxels was analyzed for each image (total volume ~1.5 ml). The raw images were filtered using a 3D median filter with a radius of 3 voxels and the image contrast was then improved using CLAHE routine in Fiji. Segmentation into water and oil phases were carried out with a hysteresis segmentation method after Vogel and Kretzschmar (1996).[17] The lower and upper threshold levels were selected individually for each image. Another median filter with a radius of 3 voxels was applied to the segmented images to remove small objects. Quantification of water droplet properties was carried out using 3D LibMorphJ plugin.[18] The water droplets in each 3D image were labelled and their size and surface area were quantified. The frequency of each droplet size was then calculated by counting voxels in each object. A total of 7 scans were used.

Bitumen mineralization to carbon dioxide

The stepwise degradation of crude oil or bitumen by fermenting and sulfate reducing bacteria, leads to a variety of different intermediates like short-chain fatty acids, alcohols, acetate, formate, and hydrogen. The final mineralization product is carbon dioxide (table S3).[19-25] This makes carbon dioxide a good reference product for biodegradation under sulfate reducing conditions.

Table S3 Reaction stoichiometries for syntrophic hydrocarbon degradation in petroleum reservoirs.

Substrate	Stoichiometric equation
Syntrophic hydrocarbon oxidation	
Toluene	$C_7H_8 + 7H_2O \rightarrow 3.5CH_3COO^- + 4H_2 + 3.5H^+$ [19]
Naphthalene	$C_{10}H_8 + 10H_2O \rightarrow 5CH_3COO^- + 4H_2 + 5H^+$ [19]
Hexadecane	$C_{16}H_{34} + 16H_2O \rightarrow 8CH_3COO^- + 17H_2 + 8H^+$ [19, 26, 27]

Syntrophic acetate oxidation	
Acetate	$CH_3COO^- H^+ 2H_2O \rightarrow 2CO_2 + 4H_2$ [27, 28]
Hydrocarbon degradation by sulfate reduction	
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$
Benzene	$C_6H_6 + 3H_2O + 3.75SO_4^{2-} \rightarrow 6HCO_3^- + 3.75HS^- + 2.25H^+$ [21]
Toluene	$C_7H_8 + 4.5SO_4^{2-} + 3H_2O \rightarrow 7HCO_3^- + 2.5H^+ + 4.5HS^-$ [24]
Decane	$C_{10}H_{22} + 7.75SO_4^{2-} + 5.5H^+ \rightarrow 10HCO_3^- + 7.75H_2S + H_2O$ [25]
Naphthalene	$C_{10}H_8 + 6SO_4^{2-} + 6H_2O \rightarrow 10HCO_3^- + 6HS^- + 4H^+$ [21]
2-methylnaphthalene	$C_{11}H_{10} + 6.75SO_4^{2-} + 6H_2O \rightarrow 11HCO_3^- + 6.75HS^- + 4.25H^+$ [21]
Phenanthrene	$C_{14}H_{10} + 8.25SO_4^{2-} + 9H_2O \rightarrow 14HCO_3^- + 8.25HS^- + 5.75H^+$ [21]
Hexadecane	$C_{16}H_{34} + 12.25SO_4^{2-} + 8.5H^+ \rightarrow 16HCO_3^- + 12.25H_2S + H_2O$ [22, 23]

REFERENCES

1. Meckenstock, R. U.; von Netzer, F.; Stumpp, C.; Lueders, T.; Himmelberg, A. M.; Hertkorn, N.; Schmitt-Kopplin, P.; Harir, M.; Hosein, R.; Haque, S., Water droplets in oil are microhabitats for microbial life. *Science* **2014,** *345*, 673-676.
2. Himmelberg, A. Life in pitch-Bacteria in a natural oil emitting lake help to understand anaerobic biodegradation of polycyclic aromatic hydrocarbons. PhD-Thesis, Technical University of Munich, **2019**.
3. Lueders, T.; Manefield, M.; Friedrich, M. W., Enhanced sensitivity of DNA-and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **2004,** *6*, 73-78.
4. Gabor, E. M.; de Vries, E. J.; Janssen, D. B., Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiol Ecol* **2003,** *44*, 153-163.
5. Zeugin, J. A.; Hartley, J. L., Ethanol precipitation of DNA. *Focus* **1985,** *7*, 1-2.
6. Crouse, J.; Amorese, D., Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. *Focus* **1987,** *9*, 3-5.
7. Sambrook, H., Molecular cloning: a laboratory manual. Cold Spring Harbor, NY. **1989**.
8. Pannekens, M.; Voskuhl, L.; Meier, A.; Müller, H.; Haque, S.; Frösler, J.; Brauer, V.; Meckenstock, R., Densely populated water droplets in heavy oil seeps. *Appl Environ Microbiol* **2020,** *86*, 1-12.
9. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F. O., Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **2013,** *41*, e1-e1.
10. Neu, T. R.; Swerhone, G. D.; Lawrence, J. R., Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. *Microbiology* **2001,** *147*, 299-313.

11. Peltola, M.; Neu, T. R.; Raulio, M.; Kolari, M.; Salkinoja-Salonen, M. S., Architecture of Deinococcus geothermalis biofilms on glass and steel: a lectin study. *Environ Microbiol* **2008,** *10*, 1752-1759.
12. Mackowiak, M. *Interaction of Enteric Viruses with Aquatic Biofilms in the Urban Water Cycle*. PhD-thesis, University of Duisburg-Essen, **2019**.
13. Thyssen, C. Biofilm formation by the manganese-oxidizing bacterium *Leptothrix discophora strain SS-1* and corrosion of stainless steel. PhD-thesis, University of Duisburg-Essen, **2018**.
14. Strathmann, M.; Wingender, J.; Flemming, H.-C., Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*. *J Microbiol Methods* **2002,** *50*, 237-248.
15. Shiina, N.; Tateno, H.; Ogawa, T.; Muramoto, K.; Saneyoshi, M.; Kamiya, H., Isolation and characterization of L-rhamnose-binding lectins from chum salmon (Oncorhynchus keta) eggs. *Fish Sci* **2002,** *68*, 1352-1366.
16. Schindelin, J., Fiji: An open-source platform for biological-image analysis. *In Nat Methods*, **2019**; Vol. 9, pp 676–682.
17. Vogel, H.; Kretzschmar, A., Topological characterization of pore space in soil—sample preparation and digital image-processing. *Geoderma* **1996,** *73*, 23-38.
18. Legland, D.; Arganda-Carreras, I.; Andrey, P., MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics* **2016,** *32*, 3532-3534.
19. Gieg, L. M.; Fowler, S. J.; Berdugo-Clavijo, C., Syntrophic biodegradation of hydrocarbon contaminants. *Curr Opin Biotechnol* **2014,** *27*, 21-29.
20. Jiménez, N.; Richnow, H. H.; Vogt, C.; Treude, T.; Krüger, M., Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *J Mol Microbiol Biotechnol* **2016,** *26*, 227-242.
21. Meckenstock, R. U.; Boll, M.; Mouttaki, H.; Koelschbach, J. S.; Tarouco, P. C.; Weyrauch, P.; Dong, X.; Himmelberg, A. M., Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. *J Mol Microbiol Biotechnol* **2016,** *26*, 92-118.
22. Mbadinga, S. M.; Wang, L.-Y.; Zhou, L.; Liu, J.-F.; Gu, J.-D.; Mu, B.-Z., Microbial communities involved in anaerobic degradation of alkanes. *Int Biodeter Biodegr* **2011,** *65*, 1-13.
23. Aeckersberg, F.; Bak, F.; Widdel, F., Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch Microbiol* **1991,** *156*, 5-14.
24. Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F., Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* **1998,** *22*, 459-473.
25. Rueter, P.; Rabus, R.; Wilkest, H.; Aeckersberg, F.; Rainey, F. A.; Jannasch, H. W.; Widdel, F., Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature* **1994,** *372*, 455-458.
26. Head, I.; Larter, S.; Gray, N.; Sherry, A.; Adams, J.; Aitken, C.; Jones, D.; Rowan, A.; Huang, H.; Röling, W., Hydrocarbon degradation in petroleum reservoirs. In *Handbook of hydrocarbon and lipid microbiology*, Springer: **2010;** pp 3097-3109.
27. Jones, D.; Head, I.; Gray, N.; Adams, J.; Rowan, A.; Aitken, C.; Bennett, B.; Huang, H.; Brown, A.; Bowler, B., Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **2008,** *451*, 176-180.
28. Xu, D.; Zhang, K.; Li, B.-G.; Mbadinga, S. M.; Zhou, L.; Liu, J.-F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Simulation of *in situ* oil reservoir conditions in a laboratory bioreactor testing for methanogenic conversion of crude oil and analysis of the microbial community. *Int Biodeterior Biodegradation* **2019,** *136*, 24-33.

5 General discussion

In 2007, Kim and Crowley investigated bitumen seeps from the La Brea Tar Pits (Los Angeles, CA, USA) regarding microbial appearance. In the process, they successfully extracted DNA from the bituminous phase and proposed the presence of possible larger water pockets, which they believed could serve as microbial hotspots in these natural bitumen seeps.[1] In 2014, Meckenstock et al. found living microorganisms inhabiting tiny water droplets dispersed through the natural bitumen of the Pitch Lake in Trinidad and Tobago.[2] These accessible and undisturbed microhabitats are an exceptional opportunity to overcome the common sampling issues of petroleum reservoirs and allow to study natural occurring microbial communities in bitumen.

Therefore, the aim of this dissertation was to investigate and describe microbial life in these recently discovered microhabitats in natural bitumen to draw general conclusions for microbial life in deep subsurface petroleum reservoirs away from the oil-water transition zone (OWTZ).

5.1 Water droplets in natural bitumen reservoirs

To proof the hypothesis that inhabited microscale water droplets are a common feature in bitumen reservoirs rather than a feature exclusively occurring in the Pitch Lake, bitumen samples were taken from three different natural bitumen seeps. Samples were taken from the Pitch Lake on Trinidad, from the La Brea Tar Pits, and from an unnamed oil seep on the McKittrick oil field (McKittrick, CA, USA).[3] Through the investigations, water droplets with similar volumes of 1-10 µl could be extracted from the surface of bitumen samples of all three investigated natural bitumen seeps.[3] These findings give a strong indication that those water droplets are a generic trait in bitumen reservoirs rather than a unique phenomenon. Consequently, the question arises about the actual water distribution within the bitumen phase.

5.2 Water distribution in natural bitumen

The current concept indicates that the major fraction of water is located at the bottom of the oil-bearing leg, at the OWTZ where most of the biodegradation occurs.[4-8]

Several studies report the presence of water dispersed in petroleum reservoirs by measuring the total water content in crude oil or bitumen. However, less is known about the spatial

distribution of water inside the oil or bitumen phase and hence, the associated oil-water surface area.[2, 7, 9-13]

Therefore, computed tomography (CT) was used to representatively scan bitumen from the Pitch Lake to reveal the distribution and size of dispersed water droplets and the associated surface area.[14] Thereby, scans of a bitumen volume of 8.45 ml with CT revealed 6330 separated water droplets inside the bitumen. Most of these water inclusions (~97 %) were rather small with a volume less than 10 nl. Only 0.2 % of the present inclusions in the scans were larger than 100 nl, including the three largest water droplets in these samples with volumes of 0.42 µl, 0.55 µl, and 2.33 µl, respectively. Further, the scans revealed an average water content of 2.5 µl ml^{-1} bitumen, resulting in an average bitumen-water surface of 1.13 cm^2 ml^{-1}.[14] Even if only a minor fraction of the enclosed water droplets is large enough to accommodate microbial communities, these results indicate that the water droplets enlarge the oil-water interface in an oil reservoir and therefore presumably affect the overall biodegradation process in natural oil or bitumen reservoirs.

5.3 Microscale water droplets in natural bitumen as densely populated habitats

In 2019, based on the state of literature at that time, Pannekens et al. proposed that microorganisms could live in small water-filled rock pores, in thin water films around sand grains, or in dispersed water through the entire oil reservoir as described in the Pitch Lake in addition to the underlying OTWZ as microbial hotspot.[1, 2, 15]

To verify the hypothesis, formerly extracted water droplets from the three already mentioned bitumen seeps were examined.[3] Direct investigation of the extracted droplets by light microscopy confirmed the presence of microorganisms in water droplets of all three tested bitumen seeps and also revealed dense populations. The cell numbers differed across a broad range of 5.6×10^3 up to 1.2×10^6 cells $µl^{-1}$. The average cell numbers in the tested seeps were 1.2×10^5 cells $µl^{-1}$ (n = 10) in Pitch Lake bitumen, 2.6×10^4 cells $µl^{-1}$ (n = 10) in the McKittrick bitumen, and 4.5×10^5 cells $µl^{-1}$ (n = 10) in La Brea Tar Pit bitumen, respectively. In addition, the light microscopy results were confirmed by fluorescence microscopy of filtered and DAPI-stained water droplets, extracted from Pitch Lake bitumen. The average cell number in all examined water droplets was $2.0 \times 10^5 \pm 3.6 \times 10^4$ cells $µl^{-1}$, which is much higher compared to mixed production waters of other oil fields, but in the same magnitude of petroleum contaminated subsurface soil ecosystems.[16-18]

The obtained micrographs indicate a multitude of different morphologies such as rods, diplobacilli, cocci, diplococci, or filamentous microorganisms. Interestingly, the majority of

the extracted and examined water droplets contained microbial cells and only a few examined water droplets were void of cells.[3]

To get more insights about the microorganisms present in these microscale water droplets, *in situ* staining was used to visualize the microbial cells inside their habitat. Thus, a Syto9-staining solution was added directly into the enclosed droplets, followed by CLSM analysis. Thereby, microbial cells inside enclosed water droplets could be visualized. Furthermore, new information about the spatial arrangement of microorganisms inside the water droplets were gained. The *in situ* measurements confirmed that only larger water droplets contained microorganisms, which were mostly densely populated, indicating that a certain amount of water is required.[3] Three-dimensional CLSM scans showed that the majority of the present microbial cells seemed to be attached or located at the bitumen-water interface around the droplet. Planktonic cells, however, were observed as well.[3] This findings indicate syntrophic interactions between primary and secondary hydrocarbon degraders to overcome possible mass transfer limitations.[5]

The results show that all investigated bitumen reservoirs contained microbial inhabited water droplets. This is further supported by other studies, in which DNA has been extracted directly from bitumen, which possibly originated from similar or former water enclosures in these bitumen samples.[12, 19] In addition, it could be shown that these water droplets are not just lightly inhabited but rather are densely populated by microbial cells. Interestingly, the average cell numbers of the tested bitumen seeps only differed by a factor of ten, indicating similarities, even though the tested bitumen seeps are hundreds to thousand kilometers away from each other. However, due to the small set of tested bitumen reservoirs, these findings do not provide absolute evidence for a generic trait of bitumen reservoirs, but proof that microbial life and degradation can occur through the entirety of an oil reservoir, if water is present.

5.4 Natural microbial communities in bitumen reservoirs

Many studies have described the presence of microorganisms in formation or production waters of numerous deep subsurface oil reservoirs.[11, 20-22] However, during the pumping process the foamy water and oil containing samples are intensively mixed before sampling.[10, 11] Furthermore, the anthropogenic injection of hot steam, production-, or seawater into subsurface petroleum reservoirs through the extraction process causes changes in the natural community composition of present microorganisms. Water flooding even leads to the introduction of external microorganisms to the natural microbiome.[10, 13, 20-24] Therefore,

Pannekens et al. (2019) suggest that the community analysis of production waters does not appropriately represent partially separated natural communities, but mostly accounts for the entirety of the present microbiome in reservoirs.[15]

To determine the community composition of undisturbed microbial communities in petroleum reservoirs, DNA was extracted from water droplets extracted from the three investigated bitumen seeps. After DNA extraction, 16S gene sequencing was performed. The results revealed complex and diverse microbial communities in water droplets of each analyzed bitumen seep. Although, the bitumen reservoirs are spatially distinct, the bitumen seeps shared about 16 % of the received OTUs (total of 558 OTUs). Thereby, each individual water droplet accommodated between 64 up to 316 different OTUs. The 10 most abundant OTUs in each bitumen seep only accounted for 8.4 % (Pitch Lake), 4.5 % (McKittrick), and 7.5 % (La Brea Tar Pits) of the entire community, showing a high diversity within the microbial community. This was further supported by high Simpson diversity indices, indicating water droplets containing diverse, but evenly distributed microbial communities.[3] The results show that the microbial communities in these tiny microhabitats are not dominated by one type of microorganism. This agrees with the current concept of syntrophic consortia of microorganisms being involved in biodegradation of petroleum.[25-31] Calculated Bray-Curtis dissimilarities for each induvial droplet community revealed that the community composition was more similar within each tested bitumen reservoir compared to the others. Thereby, the Pitch Lake exclusively contained 31 OTUs, McKittrick oil seep 79 OTUs, and the La Brea Tar Pits 147 OTUs. On the other hand, 8 OTUs were not only present in each tested seep, but present in 97-100 % of all analyzed water droplets, building a core community that accounted for 3.2 % (Pitch Lake), 1.2 % (McKittrick), and 1,6 % (La Brea) of each community.[3]

Anaerobic and facultatively anaerobic members of the most abundant families *Hydrogenophilaceae*, *Porphyromonadaceae*, *Comamonadaceae*, *Methanotrichaceae*, *Pseudomonadaceae*, *Clostridiales_Incertae_Sedis_XI*, *Desulfobulbaceae*, *Syntrophobacteraceae*, *Syntrophorhabdus*, *Deferribacteraceae*, *Synergistaceae*, *Petrotogaceae*, *Syntrophaceae*, and *Anaerolineaceae* and the present phylum of Atribacteria were previously reported for other petroleum reservoirs around the world, further indicating similarities in the degradation processes of different petroleum reservoirs.[10, 22, 32-46]

The highly selective character of oil reservoirs (e. g. water availability, pressure, temperature, salinity, toxicity) possibly lead to highly adapted and similar microbial community compositions. The fact that all three tested reservoirs share several OTUs of microorganisms,

which had previously been described to occur in other petroleum reservoirs, indicates comparable, but not completely identical, microbial processes amongst different petroleum reservoirs. This study underlines the importance of analyzing undisturbed samples to observe separated and varying microbial communities.

5.5 Biofilm formation in bitumen reservoirs

Biofilms are the dominating form of life in most habitats around the world. The biofilm mode of life provides protection against various environmental stressors, helps to overcome mass transfer limitations, and supports interspecies electron transfer.[47-51] Biofilm formation has previously been observed in the presence of petroleum and at crude oil or bitumen contaminated sites.[52-56] Therefore, it was assumed that microorganisms inhabiting oil reservoirs are mainly arranged in biofilms and that biofilms are the predominant form of life in oil reservoirs.[15]

Thus, in 2020, the spatial arrangement of microbial cells enclosed in water droplets dispersed was investigated in bitumen from all three tested bitumen seeps. Two- and three-dimensional micrographs of Syto9-stained water droplets revealed that most of the present cells are located at the water-bitumen interface surrounding the droplet.[3] Depending on the definition of a biofilm, this is a first indication for biofilm formation at the oil-water interface.[48, 57-59] However, the water volume of the droplets was artificially increased by the addition of staining solution, which possibly affected these results.

Further evidence for biofilm formation in water droplets was attained in Pannekens et al. (2021) by incubating untreated natural bitumen covered by artificial saltwater medium (based on water extracted from bitumen).[14] During the incubation period, microorganisms previously enclosed in the bitumen phase reached the overlaying water layer and formed a rather stable and active microbial community. After 945 days of incubation, 16S rRNA gene sequence analysis from the overlaying water phase and cells attached to the bitumen surface revealed different microbial community compositions between planktonic and sessile cells.[14] The aqueous phase was mainly dominated by bacteria of the order *Desulfobacterales* (29.2 ± 11.5 %), members of the candidate phylum JS1 (17.6 ± 10.1 %), and the genera *Lentimicrobium* (10.8 ± 5.4 %), *Thermovirga* (9.2 ± 2.5 %), and *Defluviitoga* (4.9 ± 0.8 %). These microorganisms were also present at the bitumen-water interface, but in lower abundances (*Desulfobacterales* 9.6 ± 1.4 %; candidate class *JS1* 7.1 ± 0.9 %; *Lentimicrobium* 4.6 ± 2.4 %, *Thermovirga* 3.5 ± 0.9 %, *Defluviitoga* 4.1 ± 1.0 %). On the other hand, the microbial community on the bitumen surface was dominated by members of the family

Deferribacteraceae (17.9 ± 3.0 %, in contrast to 0.04 ± 0.03 % in the aqueous phase) and the order *Gammaproteobacteria* (13.8 ± 3.7 %, in contrast to 0.02 ± 0.02 % in the aqueous phase). The same trend was observed for members of the phylum Halobacterota and the candidate phylum Atribacteria.[14]

To demonstrate the formation of EPS-like structures on the bitumen surface in the microcosms, a mixture of Syto9 (cell visualization) and seven fluorescently-labelled lectins (specific binding to EPS sugars) were used to cover a broad range of target sugars known to be part of EPS.[60-64] The combined Syto9-lectin staining showed the presence of green and red fluorescent structures (red mostly around green aggregates) directly on the bitumen surface . These structures did not occur in any control sample and did not exhibit any auto-fluorescence without staining.[14] Although some lectins are known to show unspecific binding to non-target molecules, these findings further indicate biofilm formation by EPS-embedded microbial cells on the bitumen surface.[62] Combined with community composition analysis and *in situ* micrographs of water droplets enclosed in bitumen, strong evidence for biofilm formation at the bitumen-water interface under natural and close to natural conditions has been gained.[3, 14]

5.6 Microbial activity and degradation rates in bitumen reservoirs

The presence of microorganisms in many petroleum reservoirs with temperatures up to 82 °C is an indication for microbial life within deep subsurface reservoirs.[10, 65] Other studies showed microbial growth in the presence of bitumen when incubated in medium.[54-56] However, little is known about the actual processes, the microbial activity, and degradation rates in deep subsurface petroleum reservoirs.[66] The current model predicts that most of the biodegradation occur at the OWTZ.[5-7]

The presence of living microorganisms in water droplets dispersed through the bitumen of the Pitch Lake raises two questions: (i) How active are these microorganisms being located far away from the OWTZ, and (ii) what is their contribution to the overall biodegradation process?[2, 3] In 2019, Pannekens et al. proposed that dispersed and inhabited water droplets and enclosures enlarge the overall oil-water interface and therefore might have a significant impact on the entire degradation process in a reservoir.[15] This claim was supported by Larter et al. (2003), describing gradual biodegradation of hydrocarbons starting from the OWTZ, which promotes the idea of active biodegradation in dispersed water within the oil phase.[7, 66] In order to prove that the enclosed microorganisms are alive and active, cells extracted from droplets of the previously mentioned three sample sites (Pitch Lake, La Brea Tar Pits, and McKittrick) were examined using the viability markers membrane integrity and ATP content.

The presence of membrane integrity is not a direct proof, but rather a prerequisite of microbial activity. LIVE/DEAD staining was performed to differentiate between membrane-intact and membrane-damaged cells in extracted water droplets. The results showed that 46 % of 197 counted cells from Pitch Lake bitumen, 56 % of 1564 counted cells in La Brea bitumen, and 57 % of 1394 counted cells in McKittrick bitumen exhibited intact cell membranes and were thus considered alive. On average, approximately half of the investigated cells (53 %) appeared to be intact.[3] Different factors such as false staining of Gram positive cells, oxygen stress during the staining procedure, and staining of damaged but viable cells could have led to an overestimation of membrane-damaged cells. Therefore, an even higher portion of present cells could be alive.[67-70]

Another proof of microbial activity is the presence of the energy carrier ATP. Since extracellular ATP is rapidly consumed in the presence of intact cells or enzymes and only restored by living microorganisms, it can be used as a valid proof for metabolic activity of living cells.[71, 72] Thus, the concentration of ATP were determined in water droplets extracted from the bitumen of the three tested bitumen reservoirs. Thereby, it was possible to quantify ATP in most of the examined water droplets, with variating concentrations between the different bitumen reservoirs. The determined average ATP concentrations were 21.8 pM in droplets from the Pitch Lake, 194.8 pM in droplets of the McKittrick oil field, and 492.2 pM in droplets from the La Brea Tar Pits.[3] Since ATP was found to be present in most of the tested water droplets, these microhabitats were shown to be inhabited by active microbial communities. This confirms that biodegradation of hydrocarbons can occur through the entire petroleum reservoir.

After establishing proof for active microorganisms inside the water droplets, it was possible to determine their impact on the overall hydrocarbon degradation in oil reservoirs by measuring natural degradation rates. This was done by applying the recently invented "reverse stable isotope labeling" in order to measure the complete mineralization of bitumen components to CO_2.[73-75] Pitch Lake bitumen was incubated in four microcosms for 945 days with an overlaying layer of brackish water medium, which was prepared according to analyzed water former extracted from Pitch Lake bitumen.

Since 9.7 ± 3.9 mM sulfate was present in water samples extracted from Pitch Lake bitumen, and for the reason that sulfate reducing bacteria (SRB) are found to be present in almost every petroleum reservoir, this study focused mainly on the production of CO_2 by sulfate reduction and possible fermentation.[14, 31, 76] Methane production was not monitored, since sulfate reduction outcompetes or inhibits methanogenesis.[56, 66, 77-79] During the

incubation, in the microcosms an average of 8.5 mM sulfate was consumed. In the same time an average of 24.5 mM CO_2 was produced, accounting for an absolute production of 2.93 mmol CO_2 on average.[14] By linking the mineralization and bitumen CT scan results, it was possible to calculate the mineralization rate per surface and time of 8.3−34.1 μmol CO_2 cm^{-2} $year^{-1}$ and the mineralization rate per bitumen volume and time of 9.4−38.6 mmol CO_2 L^{-1} bitumen $year^{-1}$.[14] In addition to the sulfate and CO_2 measurements, the cell numbers in all microcosms were monitored. During the experiment cell counts increased by approximately two orders of magnitude, ranging from $4.3 \times 10^5 \pm 3.7 \times 10^5$ cells ml^{-1} at the start of the incubation up to $1.4 \times 10^7 \pm 1.2 \times 10^6$ cells ml^{-1} after 945 days, again demonstrating activity in bitumen degrading microbial communities. Considering that biodegradation of hydrocarbons occurs over geological time scales through the entire petroleum reservoir, therefore, inhabited water droplets can expect to have a major impact on the overall degradation process in the reservoir.

5.7 General conclusion

In summary, this study confirms that inhabited, microliter-sized water inclusions in bitumen are not a phenomenon exclusively occurring in the Pitch Lake, but rather are common in natural bitumen reservoirs. It could be shown that these water droplets are densely populated microhabitats with cell densities up to 10^6 cells ml^{-1}. The microorganisms present in these droplets were shown to be alive and active within their habitat. Strong evidence for the formation of biofilms at the oil-water interface has been presented. Community analysis showed the presence of typical petroleum-degrading microorganisms and similarities in the overall community composition amongst all tested natural bitumen reservoirs, revealing a possible core community. Reverse stable isotope labeling was successfully applied to precisely monitor bitumen mineralization over time. Furthermore, the 3D scans of Pitch Lake bitumen revealed a multitude of entrapped water droplets, which enlarge the overall oil-water interface in petroleum reservoirs. The obtained mineralization rates indicate that microorganisms in dispersed water droplets have the potential to play an important role in the overall degradation process in deep subsurface petroleum reservoirs over geological times scales. This study gives new insights about life in natural bitumen reservoirs far away from the OWTZ. These findings build the foundation for further research regarding microbial ecology, syntrophic interactions, and metabolic processes in deep subsurface petroleum reservoirs.

5.8 References – General discussion

1. Kim, J. S.; Crowley, D. E., Microbial diversity in natural asphalts of the rancho la brea tar pits. *Appl. Environ. Microbiol.* **2007,** *73*, 4579-4591.

2. Meckenstock, R. U.; von Netzer, F.; Stumpp, C.; Lueders, T.; Himmelberg, A. M.; Hertkorn, N.; Schmitt-Kopplin, P.; Harir, M.; Hosein, R.; Haque, S., Water droplets in oil are microhabitats for microbial life. *Science* **2014,** *345*, 673-676.

3. Pannekens, M.; Voskuhl, L.; Meier, A.; Müller, H.; Haque, S.; Frösler, J.; Brauer, V.; Meckenstock, R., Densely populated water droplets in heavy oil seeps. *Appl. Environ. Microbiol.* **2020,** *86*, e00164-20.

4. Abbasnezhad, H.; Gray, M.; Foght, J. M., Influence of adhesion on aerobic biodegradation and bioremediation of liquid hydrocarbons. *Appl. Microbiol. Biotechnol.* **2011,** *92*, 653-675.

5. Bennett, B.; Adams, J.; Gray, N.; Sherry, A.; Oldenburg, T.; Huang, H.; Larter, S.; Head, I., 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.* **2013,** *56*, 94-105.

6. Larter, S.; Huang, H.; Adams, J.; Bennett, B.; Jokanola, O.; Oldenburg, T.; Jones, M.; Head, I.; Riediger, C.; Fowler, M., The controls on the composition of biodegraded oils in the deep subsurface: Part II—Geological controls on subsurface biodegradation fluxes and constraints on reservoir-fluid property prediction: Part I of this study was published in Organic Chemistry in 2003 (Larter et al., 2003). *Am. Assoc. Pet. Geol. Bull.* **2006,** *90*, 921-938.

7. Head, I. M.; Jones, D. M.; Larter, S. R., Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **2003,** *426*, 344.

8. Schneiker, S.; dos Santos, V. A. P. M.; Bartels, D.; Bekel, T.; Brecht, M.; Buhrmester, J.; Chernikova, T. N.; Denaro, R.; Ferrer, M.; Gertler, C.; Goesmann, A.; Golyshina, O. V.; Kaminski, F.; Khachane, A. N.; Lang, S.; Linke, B.; McHardy, A. C.; Meyer, F.; Nechitaylo, T.; Puhler, A.; Regenhardt, D.; Rupp, O.; Sabirova, J. S.; Selbitschka, W.; Yakimov, M. M.; Timmis, K. N.; Vorholter, F. J.; Weidner, S.; Kaiser, O.; Golyshin, P. N., Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nat. Biotechnol.* **2006,** *24*, 997-1004.

9. Schulze-Makuch, D.; Haque, S.; Antonio, M. R. D.; Ali, D.; Hosein, R.; Song, Y. C.; Yang, J. S.; Zaikova, E.; Beckles, D. M.; Guinan, E.; Lehto, H. J.; Hallam, S. J., Microbial Life in a Liquid Asphalt Desert. *Astrobiology* **2011,** *11*, 241-258.

10. Magot, M.; Ollivier, B.; Patel, B. K. C., Microbiology of petroleum reservoirs. *Antonie Van Leeuwenhoek* **2000,** *77*, 103-116.

11. Kobayashi, H.; Endo, K.; Sakata, S.; Mayumi, D.; Kawaguchi, H.; Ikarashi, M.; Miyagawa, Y.; Maeda, H.; Sato, K., 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.* **2012,** *113*, 204-210.

12. Ridley, C. M.; Voordouw, G., Aerobic microbial taxa dominate deep subsurface cores from the Alberta oil sands. *FEMS Microbiol. Ecol.* **2018,** *94*, fiy073.

13. Sierra-Garcia, I. N.; de Oliveira, V. M., Microbial hydrocarbon degradation: efforts to understand biodegradation in petroleum reservoirs. *Biodegradation-engineering and technology* **2013,** *10*, 55920.

14. Pannekens, M.; Voskuhl, L.; Mohammadian, S.; Köster, D.; Meier, A.; Köhne, J. M.; Kulbatzki, M.; Akbari, A.; Haque, S.; Meckenstock, R. U., Microbial Degradation Rates of Natural Bitumen. *Environ. Sci. Technol.* **2021,** *55*, 8700-8708.

15. Pannekens, M.; Kroll, L.; Müller, H.; Mbow, F. T.; Meckenstock, R. U., Oil reservoirs, an exceptional habitat for microorganisms. *New. Biotechnol.* **2019,** *49*, 1-9.

16. Orphan, V.; Taylor, L.; Hafenbradl, D.; Delong, E., Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl. Environ. Microbiol.* **2000,** *66*, 700-711.

17. Lenchi, N.; Inceoglu, O.; Kebbouche-Gana, S.; Gana, M. L.; Lliros, M.; Servais, P.; Garcia-Armisen, T., Diversity of Microbial Communities in Production and Injection Waters of Algerian Oilfields Revealed by 16S rRNA Gene Amplicon 454 Pyrosequencing. *PloS one* **2013,** *8*.

18. Long, S.; Aelion, C.; Dobbins, D. C.; Pfaender, F. K., A comparison of microbial community characteristics among petroleum-contaminated and uncontaminated subsurface soil samples. *Microb. Ecol.* **1995,** *30*, 297-307.

19. Wong, M.-L.; An, D.; Caffrey, S. M.; Soh, J.; Dong, X.; Sensen, C. W.; Oldenburg, T. B.; Larter, S. R.; Voordouw, G., Roles of thermophiles and fungi in bitumen degradation in mostly cold oil sands outcrops. *Appl. Environ. Microbiol.* **2015,** *81*, 6825-6838.

20. Ollivier, B.; Magot, M.; Cunningham-Rundles, S., *Petroleum microbiology*. ASM Press Washington, DC: 2005.

21. Li, X.-X.; Mbadinga, S. M.; Liu, J.-F.; Zhou, L.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Microbiota and their affiliation with physiochemical characteristics of different subsurface petroleum reservoirs. *Int. Biodeter. Biodegr.* **2017,** *120*, 170-185.

22. Gao, P. K.; Tian, H. M.; Wang, Y. S.; Li, Y. S.; Li, Y.; Xie, J. X.; Zeng, B.; Zhou, J. F.; Li, G. Q.; Ma, T., Spatial isolation and environmental factors drive distinct bacterial and archaeal communities in different types of petroleum reservoirs in China. *Sci Rep-Uk* **2016,** *6*.

23. Korenblum, E.; Souza, D. B.; Penna, M.; Seldin, L., Molecular analysis of the bacterial communities in crude oil samples from two Brazilian offshore petroleum platforms. *Int. J. Microbiol.* **2012,** *2012*, 8.

24. Vigneron, A.; Alsop, E. B.; Lomans, B. P.; Kyrpides, N. C.; Head, I. M.; Tsesmetzis, N., Succession in the petroleum reservoir microbiome through an oil field production lifecycle. *The ISME journal* **2017,** *11*, 2141-2154.

25. McGenity, T. J.; Folwell, B. D.; McKew, B. A.; Sanni, G. O., Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquat. Biosyst.* **2012,** *8*, 1-19.

26. Mbadinga, S. M.; Wang, L.-Y.; Zhou, L.; Liu, J.-F.; Gu, J.-D.; Mu, B.-Z., Microbial communities involved in anaerobic degradation of alkanes. *Int. Biodeter. Biodegr.* **2011,** *65*, 1-13.

27. Xu, D.; Zhang, K.; Li, B.-G.; Mbadinga, S. M.; Zhou, L.; Liu, J.-F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Simulation of *in situ* oil reservoir conditions in a laboratory bioreactor testing for methanogenic conversion of crude oil and analysis of the microbial community. *Int. Biodeterior. Biodegradation.* **2019,** *136*, 24-33.

28. Head, I.; Larter, S.; Gray, N.; Sherry, A.; Adams, J.; Aitken, C.; Jones, D.; Rowan, A.; Huang, H.; Röling, W., Hydrocarbon degradation in petroleum reservoirs. In *Handbook of hydrocarbon and lipid microbiology*, Springer: 2010; pp 3097-3109.

29. Zengler, K.; Richnow, H. H.; Rosselló-Mora, R.; Michaelis, W.; Widdel, F., Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **1999,** *401*, 266.

30. Jones, D.; Head, I.; Gray, N.; Adams, J.; Rowan, A.; Aitken, C.; Bennett, B.; Huang, H.; Brown, A.; Bowler, B., Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **2008,** *451*, 176-180.

31. Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F., Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol. Rev.* **1998,** *22*, 459-473.

32. Kano, S.; Mukaidani, T.; Hattori, Y.; Fujiwara, K.; Miyagawa, Y.; Takabayashi, K.; Maeda, H.; Okatsu, K., Diversity of indigenous anaerobes and methane conversion system

from reservoir oil by indigenous anaerobes in depleted oil fields. *J. Jpn. Petrol. Inst.* **2009,** *52*, 297-306.

33. Dahle, H.; Garshol, F.; Madsen, M.; Birkeland, N. K., Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. *Antonie Van Leeuwenhoek* **2008,** *93*, 37-49.

34. Orlygsson, J.; Kristjansson, J. K., *The Family Hydrogenophilaceae*. Springer-Verlag GmbH, Heidelberg: 2014; p 859-868.

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

36. Tamazawa, S.; Mayumi, D.; Mochimaru, H.; Sakata, S.; Maeda, H.; Wakayama, T.; Ikarashi, M.; Kamagata, Y.; Tamaki, H., 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.* **2017,** *67*, 3982-3986.

37. Willems, A., The family comamonadaceae. *The Prokaryotes: Alphaproteobacteria and betaproteobacteria* **2014**, 777-851.

38. Oren, A., The family Methanotrichaceae. *The Prokaryotes: Other Major Lineages of Bacteria and the Archaea* **2014**, 297-306.

39. Sakamoto, M., *The family porphyromonadaceae*. Springer-Verlag GmbH, Heidelberg: 2014; p 811-824.

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

41. Kuever, J., The Family Desulfobulbaceae. In *The Prokaryotes*, Springer: 2014; pp 75-86.

42. Kuever, J., The Family Syntrophobacteraceae. In *The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria*, 2014; pp 289-299.

43. Rees, G. N.; Patel, B. K.; Grassia, G. S.; Sheehy, A. J., Anaerobaculum thermoterrenum gen. nov., sp. nov., a novel, thermophilic bacterium which ferments citrate. *Int. J Syst. Evol. Microbiol.* **1997,** *47*, 150-154.

44. Sherry, A.; Gray, N.; Ditchfield, A.; Aitken, C.; Jones, D.; Röling, W.; Hallmann, C.; Larter, S.; Bowler, B.; Head, I., Anaerobic biodegradation of crude oil under sulphate-

reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int. Biodeter. Biodegr.* **2013,** *81*, 105-113.

45. Gray, N.; Sherry, A.; Grant, R.; Rowan, A.; Hubert, C.; Callbeck, C.; Aitken, C.; Jones, D.; Adams, J.; Larter, S., The quantitative significance of Syntrophaceae and syntrophic partnerships in methanogenic degradation of crude oil alkanes. *Environ. Microbiol.* **2011,** *13*, 2957-2975.

46. Liu, Y.-F.; Qi, Z.-Z.; Shou, L.-B.; Liu, J.-F.; Yang, S.-Z.; Gu, J.-D.; Mu, B.-Z., Anaerobic hydrocarbon degradation in candidate phylum 'Atribacteria'(JS1) inferred from genomics. *The ISME journal* **2019,** *13*, 2377-2390.

47. Kouzuma, A.; Kato, S.; Watanabe, K., Microbial interspecies interactions: recent findings in syntrophic consortia. *Front. Microbiol.* **2015,** *6*.

48. Flemming, H.-C.; Wuertz, S., Bacteria and archaea on Earth and their abundance in biofilms. *Nat. Rev. Microbiol.* **2019,** *17*, 247-260.

49. Flemming, H.-C., The perfect slime. *Colloids Surf B* **2011,** *86*, 251-259.

50. Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P., Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004,** *2*, 95-108.

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

52. Obuekwe, C. O.; Al-Zarban, S. S., Bioremediation of crude oil pollution in the Kuwaiti desert: The role of adherent microorganisms. *Environ, Int,* **1998,** *24*, 823-834.

53. Mehdi, H.; Giti, E., Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. *Int. Biodeterior.* **2008,** *62*, 170-178.

54. Ait-Langomazino, N.; Sellier, R.; Jouquet, G.; Trescinski, M., Microbial degradation of bitumen. *Experientia* **1991,** *47*, 533-539.

55. Golby, S.; Ceri, H.; Gieg, L. M.; Chatterjee, I.; Marques, L. L.; Turner, R. J., Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. *FEMS Microbiol. Ecol.* **2012,** *79*, 240-250.

56. Wolf, M.; Bachofen, R., Microbial legradation of bitumen. *Experientia* **1991,** *47*, 542-548.

57. Vert, M.; Doi, Y.; Hellwich, K. H.; Hess, M.; Hodge, P.; Kubisa, P.; Rinaudo, M.; Schue, F., Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). *Pure. Appl. Chem.* **2012,** *84*, 377-408.

58. Persat, A.; Nadell, C. D.; Kim, M. K.; Ingremeau, F.; Siryaporn, A.; Drescher, K.; Wingreen, N. S.; Bassler, B. L.; Gitai, Z.; Stone, H. A., The Mechanical World of Bacteria. *Cell* **2015,** *161*, 988-997.

59. Flemming, H.-C.; Baveye, P.; Neu, T. R.; Stoodley, P.; Szewzyk, U.; Wingender, J.; Wuertz, S., Who put the film in biofilm? The migration of a term from wastewater engineering to medicine and beyond. *NPJ Biofilms Microbiomes* **2021,** *7*, 1-5.

60. Peltola, M.; Neu, T. R.; Raulio, M.; Kolari, M.; Salkinoja-Salonen, M. S., Architecture of Deinococcus geothermalis biofilms on glass and steel: a lectin study. *Environ. Microbiol.* **2008,** *10*, 1752-1759.

61. Thyssen, C. *Biofilm formation by the manganese-oxidizing bacterium Leptothrix discophora strain SS-1 and corrosion of stainless steel*. PhD-thesis, University of Duisburg-Essen, 2018.

62. Strathmann, M.; Wingender, J.; Flemming, H.-C., Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of Pseudomonas aeruginosa. *J. Microbiol. Methods.* **2002,** *50*, 237-248.

63. Neu, T. R.; Swerhone, G. D.; Lawrence, J. R., Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. *Microbiology* **2001,** *147*, 299-313.

64. Shiina, N.; Tateno, H.; Ogawa, T.; Muramoto, K.; Saneyoshi, M.; Kamiya, H., Isolation and characterization of L-rhamnose-binding lectins from chum salmon (Oncorhynchus keta) eggs. *Fish. Sci.* **2002,** *68*, 1352-1366.

65. Bernard, F.; Connan, J.; Magot, M. In *Indigenous microorganisms in connate water of many oil fields: a new tool in exploration and production techniques*, SPE Annual Technical Conference and Exhibition, 1992; OnePetro: 1992.

66. Larter, S.; Wilhelins, A.; Head, I.; Koopmans, M.; Aplin, A.; Di Primio, R.; Zwach, C.; Erdmann, M.; Telnaes, N., The controls on the composition of biodegraded oils in the deep subsurface - part 1: biodegradation rates in petroleum reservoirs. *Org. Geochem.* **2003,** *34*, 601-613.

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

68. Amor, K. B.; Breeuwer, P.; Verbaarschot, P.; Rombouts, F. M.; Akkermans, A. D.; De Vos, W. M.; Abee, T., Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead Bifidobacterium cells during bile salt stress. *Appl. Environ. Microbiol.* **2002,** *68*, 5209-5216.

69. Davey, H. M.; Hexley, P., Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. *Environ. Microbiol.* **2011,** *13*, 163-171.

70. Shi, L.; Günther, S.; Hübschmann, T.; Wick, L. Y.; Harms, H.; Müller, S., Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry A* **2007,** *71*, 592-598.

71. Karl, D. M., Cellular nucleotide measurements and applications in microbial ecology. *Microbiol. Rev.* **1980,** *44*, 772-774.

72. Cowan, D. A.; Casanueva, A., Stability of ATP in Antarctic mineral soils. *Polar Biol.* **2007,** *30*, 1599-1603.

73. Dong, X.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U., Quantification of microbial degradation activities in biological activated carbon filters by reverse stable isotope labelling. *AMB Express* **2019,** *9*, 109.

74. Dong, X.; Jochmann, M. A.; Elsner, M.; Meyer, A. H.; Bäcker, L. E.; Rahmatullah, M.; Schunk, D.; Lens, G.; Meckenstock, R. U., Monitoring microbial mineralization using reverse stable isotope labeling analysis by mid-infrared laser spectroscopy. *Environ. Sci. Technol.* **2017,** *51*, 11876-11883.

75. Schulte, S.; Köster, D.; Jochmann, M.; Meckenstock, R., Applying reverse stable isotope labeling analysis by mid-infrared laser spectroscopy to monitor BDOC in recycled wastewater. *Sci. Total. Environ.* **2019,** *665*, 1064-1072.

76. Widdel, F.; Rabus, R., Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **2001,** *12*, 259-276.

77. Jiménez, N.; Richnow, H. H.; Vogt, C.; Treude, T.; Krüger, M., Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *J. Mol. Microbiol. Biotechnol.* **2016,** *26*, 227-242.

78. Muyzer, G.; Stams, A. J., The ecology and biotechnology of sulphate-reducing bacteria. *Nat, Rev, Microbiol,* **2008,** *6*, 441-454.

79. Kuever, J.; Rainey, F. A.; Widdel, F., Desulfobacterales ord. nov. *Bergey's Manual of Systematics of Archaea and Bacteria* **2015**, 1-2.

6 Appendix

6.1 Publication list

6.1.1 Peer-reviewed publications

Voskuhl, L.; Akbari, A.; Müller, H.; **Pannekens, M.**; Brusilova, D.; Dyksma, S.; Haque, S.; Graupner, N.; Dunthorn, M.; Meckenstock, R. U.; Brauer, V. S., Indigenous microbial communities in oil show a threshold response to salinity. Submitted to *FEMS Ecology* **2021.**

Schulze-Makuch, D.; Lipus, D.; Arens, F.; Baqué, M.; Bornemann, T.; de Vera, JP.; Flury, M.; Frösler, J.; Heinz, J.; Hwang, Y.; Kounaves, S. P.; Mangelsdorf, K.; Meckenstock, R. U.; **Pannekens, M.**; Probst, A. J.; Sáenz, J. S.; Schirmack, J.; Schloter, M.; Schmitt-Kopplin, P.; Schneider, B.; Uhl, J.; Vestergaard, G.; Valenzuela, B.; Zamorano, P.; Wagner, D., Microbial Hotspots in Lithic Microhabitats Inferred from DNA Fractionation and Metagenomics in the Atacama Desert. *Microorganisms* **2021**, 9, 1038

Pannekens, M.; Voskuhl, L.; Mohammadian, S.; Köster, D.; Meier, A.; Köhne, J. M.; Kulbatzki, M.; Akbari, A.; Haque, S.; Meckenstock, R. U., Microbial degradation rates of natural bitumen. *Environ Sci Technol* **2021,** 55, 8700-8708.

Pannekens, M.; Voskuhl, L.; Meier, A.; Müller, H.; Haque, S.; Frösler, J.; Brauer, V. S.; Meckenstock, R.U., Densely populated water droplets in heavy oil seeps. *Appl Environ Microbiol* **2020**, 86, e00164-20.

Pannekens, M.; Kroll, L.; Müller, H.; Mbow, F. T.; Meckenstock, R. U., Oil reservoirs, an exceptional habitat for microorganisms. *New Biotechnol* **2019**, 49, 1-9.

6.1.2 Poster presentations

Arens, F.; Airo, A.; Neumann, T.; **Pannekens, M.**; Meckenstock, R. U.; Scharfe, M.; Kaupenjohann, M.; Schulze-Makuch, D., Identifying New Soil Microhabitats in the Hyperarid Atacama Desert, Chile. 19th EANA Astrobiology Conference, Orléans, France **2019.**

Pannekens, M.; Meier, A.; Meckenstock, R. U., Active microorganisms in oil reservoirs. Microbiology and Infection 2017 – 5th Joint Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association for General and Applied Microbiology (VAAM), Würzburg, Germany **2017.**

Kuklinski, A.; Thyssen, C.; **Pannekens, M.**; Holuscha, D.; Fürbeth, W.; Sand, W., Corrosion protection for metals by analogues of extracellular polymeric substances. Proceedings of EUROCORR 2012, Istanbul, Turkey **2012.**

6.1.3 Oral presentations

Pannekens, M., Leben im Bitumen. Neujahrskolloquim der Fakultät für Chemie und des Ortsverbandes Essen-Duisburg der GDCh, Universität Duisburg-Essen **2019.**

Pannekens, M., A new concept for microbial life in oil reservoirs. Hans-Curt Flemming lecture, University Duisburg-Essen **2018.**

6.2 Publication usage permissions

6.2.1 Usage permit for Pannekens et al., 2019

Oil reservoirs, an exceptional habitat for microorganisms

Author: Mark Pannekens,Lisa Kroll,Hubert Müller,Fatou Tall Mbow,Rainer U. Meckenstock
Publication: New Biotechnology
Publisher: Elsevier
Date: 25 March 2019

© 2018 The Authors. Published by Elsevier B.V.

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: https://www.elsevier.com/about/our-business/policies/copyright#Author-rights

BACK CLOSE WINDOW

6.2.2 Usage permit for Pannekens et al., 2020

AMERICAN SOCIETY FOR MICROBIOLOGY

Densely Populated Water Droplets in Heavy-Oil Seeps

Author: M. Pannekens,L. Voskuhl,A. Meier,H. Müller,S. Haque,J. Frösler,V. S. Brauer,R. U. Meckenstock
Publication: Applied and Environmental Microbiology
Publisher: American Society for Microbiology
Date: May 19, 2020

Copyright © 2020, American Society for Microbiology

Creative Commons

This is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

You are not required to obtain permission to reuse this article.

6.2.3 Usage permit for Pannekens et al., 2021

Dear Mark Pannekens,

Your permission requested is granted and there is no fee for this reuse.

In your planned reuse, you must cite the ACS article as the source, add this direct link: <https://pubs.acs.org/doi/10.1021/acs.est.1c00596>, and include a notice to readers that further permissions related to the material excerpted should be directed to the ACS.

Please note: he CC-BY-ND-NC license does not allow third parties to create derivative works such as translations and only permits other types of use for noncommercial purposes. For more details on the Creative Commons license, please visit the Creative Commons website: www.creativecommons.org.

Please do not hesitate to contact me if you need any further assistance.

Regards,

[redacted]
ACS Customer Services & Information
https://help.acs.org

Case Info:

Case Number : CSCSI0004648

Created On: 06-25-2021 10:17:43 AM EDT

Cite this: *Environ. Sci. Technol.* 2021, 55, 13, 8700−8708
Publication Date: June 25, 2021
https://doi.org/10.1021/acs.est.1c00596
Copyright © 2021 The Authors. Published by American Chemical Society
RIGHTS & PERMISSIONS

6.3 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

6.4 Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

“Natural bitumen is an extraordinary habitat for microorganisms”

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, 29.10.2021

Mark Pannekens