The Influence of Elevated Pressure and Hydrocarbon Input on the Deep Sea Microbial Community of the Gulf of Mexico

Vom Promotionsausschuss der
Technischen Universität Hamburg
zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von
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aus
Mannheim

2019

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Tag der mündlichen Prüfung:

21.11.2019

Für G & H

Content

List of abbreviations

approx.	approximately
assA	alkylsuccinate synthase
API	American Petroleum Institute
ASW	artificial seawater
BP	British Petroleum
bp	base pairs
bsl.	below sea level
bssA	benzylsuccinate synthase alpha subunit
BTEX	benzene, toluene, ethylbenzene, xylene
C	carbon
CA / PES	cellulose acetate / polyethersulfone
CFU	colony forming unit
CoA	Coenzyme A
C-IMAGE	Center for Integrated Modeling and Analysis of Gulf Ecosystems
cm^3	cubic centimeter
d.oil	dispersed oil treatment (dispersant + crude oil)
disp.	dispersant alone treatment
DNA	deoxyribonucleic acid
DOR	dispersant to oil ratio
DOSS	dioctylsulfosuccinate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DWH	Deepwater Horizon
e.g.	for example (lat. exempli gratia)
EPS	extracellular polysaccharide
FSW	filtered seawater
g	gram
g/L	gram per liter
GC-MS	gas chromatography-mass spectrometry
GoM	Gulf of Mexico
GoMRI	Gulf of Mexico Research Initiative
h	hour
km	kilometer
kg	kilogram

L	liter
LB	Luria Bertani
log	logarithm
(m)M	(milli)molar
m	meter
MA	marine agar
MM2	minimal mineral medium
(s/p)MMO	solubilized or particulate methane monooxygenase
mL	milliliter
mM	millimolar
MPa	megapascal (10^6 x kg x m^{-1} x s^{-2})
µ	growth rate
µL	microliter
µm	micrometer
n	replicate number
ng/µL	nanogram per microliter
nm	nanometer
OD	optical density
OLS	ordinary least square
OTU	operational taxonomic unit
p	probability value
PAH	polycyclic aromatic hydrocarbon
PCR	Polymerase Chain Reaction
R.	*Rhodococcus*
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
rpm	revolutions per minute
R/V	research vessel
SD	standard deviation
sp(p).	species singular(/plural)
sec	second
t	metric tons
TAE	tris-acetate-EDTA
TUHH	Hamburg University of Technology
U/µL	Unit per microliter

UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

1. Introduction

1.1. Microbial Ecology of the Deep Sea: Upper Sediment and Deep Sea Pelagic Microbial Communities

- **Deep Sea Ecosystem**

The deep sea is commonly defined as the part of the oceans where no or little light penetrates to support phototrophic life anymore, which is on average below 200 m of water depth. This region (aphotic zone) is covering 95 % of the seabed and is the largest habitat on our planet (67 %). With temperatures close to freezing (-1 °C to 4 °C, extremes of -2 °C and >150 °C), increasing hydrostatic pressure (1.0 MPa per 100 m of depth), and limited availability of organic matter (Jørgensen and Boetius, 2007), the deep sea is considered an extreme environment. By volume, dark and cold conditions dominate the oceans environment and are common for our planet. Nevertheless, only 5 % of this region have been explored in detail. It has been estimated that only 0.001 % of the whole biodiversity has been sampled (Corinaldesi, 2015). Life is documented everywhere in the deep sea: from sediments in the deepest trenches (10.000 m below sea level (bsl.), Zobell and Morita 1957) to the deep biosphere up to 2 – 4 km below the seafloor, where the only limit to life is the geothermal gradient increasing temperatures with depth (Jørgensen and Boetius, 2007).

All life forms require a source of energy and carbon. Thus, microbial primary production of the oceans is controlled by the availability of organic substrates. With increasing depth, photosynthesis becomes less important in the deep sea. Therefore, the availability of electron donors and acceptors (Froelich, 1979) is mostly limited to sinking particulate organic matter into the deep biosphere, such as marine snow and fecal pellets (Boetius and Damm, 1998). This leads to a heterogeneous distribution of microbes associated with the organic matter (Orcutt et al., 2011).

- **Marine Pelagic Habitat**

The pelagic zone is the water column of the open oceans and consists of the photic (< 200 m bsl.) and the aphotic zone (> 200 m bsl.). The latter is henceforth called the deep sea. The average numbers of prokaryotic cells drop from about 10^5 to 10^6 cells mL^{-1} in the photic zone to 10^3 to 10^5 cells mL^{-1} in the deep sea (Nagata et al., 2000, Reinthaler, 2006). Interestingly, there is an increase in cell density within several hundred meters above the bottom of the sea (Nagata et al., 2000). Bacterial cells in the deep sea occur more often free-living than attached to particles (Orcutt et al., 2011).

The paradigm “everything is everywhere, the environment selects” (Baas-Becking, 1934) is supported by the general trend that the microbial diversity increases with depth in the water column (Orcutt et al., 2011), while cell numbers drop. Hewson et al. (2006) found that microbial communities from the same depth of different oceans are more similar to each other than communities from the same ocean at different depths. With an increasing water depth, the proportion of archaea in the prokaryotic community is increasing (Karner et al., 2001). According to research on the 16S rRNA gene clone libraries, common bacterial representatives of the aphotic pelagic zone are Gammaproteobacteria, including *Colwellia*, *Shewanella*, *Alteromonas*, and *Pseudoalteromonas* relatives (Zaballos, 2006). King et al. (2013) sampled the northern Gulf of Mexico (GoM) prior to the Deepwater Horizon (DWH) oil spill and found SAR11 Alphaproteobacteria and Bacteroidetes dominating the communities above 100 m of depth. Alpha- to Gammaproteobacteria ratios were above 1.7. Below 100 m this ratio dropped to < 1 and Thaumarchaeota, Firmicutes and Deltaproteobacteria became more dominant.

- **Marine Sediment**

Investigation of marine sediments revealed that the top 10 cm of the sediments worldwide are inhabited by at least 2.9×10^{29} prokaryotic cells (Kallmeyer et al., 2012), which accounts for half the cells of the ocean and for 5 – 15 % of Earth’s microbial biomass (Orcutt et al., 2011). The challenges of microbial life in the deep sea sediments are the availability of organic matter and presence of electron acceptors, while oxygen is consumed slowly and it can diffuse up to several meters down into the sediment (Fischer et al., 2009). With increasing depth of the sediment and a depletion of oxygen, alternative electron acceptors (nitrate, manganese, ferric iron and sulfate reduction) become dominant in metabolic pathways. Cell densities in deep sea sediments average at about 10^6 to 10^7 cells per cm^3 (Parkes et al., 2000), but are shown to vary by five orders of magnitude, according to the sites sedimentation rate and distance to the shore line (Kallmeyer et al., 2012).

The archaeal diversity in the oxic deep sea sediments, in general, is represented by high abundances of Crenarchaeota and Euryarchaeota (Giovannelli et al., 2013, Jørgensen and Boetius, 2007). While the bacterial assemblages are dominated by Proteobacteria (Alpha, Delta, Gamma), Acidobacteria, Actinomycetes, Chloroflexi, and Planctomycetes (Orcutt et al., 2011, Zinger et al. 2011, Parkes et al. 2014).

- **Natural Seeps in the Gulf of Mexico**

Natural oil seeps are frequently occurring (De Beukelaer et al., 2003, Solomon et al., 2009, MacDonald, 2011) in the Gulf of Mexico and are estimated to leak 2.5 - 9.6 x 10^4 t of oil annually (Kvenvolden, 2003, MacDonald et al., 2015). The majority of the 914 seep zones (68 %) detected via satellite imaging in 2010 (MacDonald et al., 2015) are located in the northwest of the GoM. Due to frequent presence of crude oil in the water column, it is assumed that the microbial communities indigenous to these natural seep sites are adapted to the regular occurring input of hydrocarbons as carbon and energy source (Hazen et al., 2010, Kleindienst et al., 2015).

1.2. Deepwater Horizon (DWH)

On April 20th 2010, the oil-drilling platform Deepwater Horizon (DWH) exploded, which took the life of 11 crew members and injured 17. The platform was situated above the continental slope, which forms the Mississippi Canyon in the northern Gulf of Mexico. It sank 2 days after the explosion. Oil should have been pumped from the well MC-252 located above the Macondo oil field. The depth of the well, at about 1500 m bsl., makes this catastrophic series of events unique up to date. As a consequence of the explosion, crude oil and natural gases flowed uncontrolled into the deep sea environment over a period of 87 days until it was possible to seal the well head. The total amount of hydrocarbons released, until the capping of the well, was estimated to be 650 – 794 million liters of crude oil and 1.7 x 10^8 kg of natural gases and liquids (C_1-C_5) (McNutt et al., 2012, Reddy et al., 2012), not including the crude oil which was recovered by emergency measures taken by BP to capture the crude oil. Budgeting all of the released oil, 17 % were directly recovered from the well, 16 % were chemically dispersed, 5 % were burned at the surface, 3 % were skimmed, while 13 % were naturally dispersed, 24 % evaporated and dissolved. However, there was still a gap in the estimations of 22 % of the crude oil, which have possibly been recovered in beach cleaning activities, remained in the water column, settled at the seafloor or has been degraded by microorganisms (Ramseur, 2010). Among others, one measure to recover the released oil by British Petroleum (BP) was to apply oil-dispersing agents, Corexit EC9500A and Corexit EC9527A. The exact composition of Corexit is not known, but the ingredient most toxic to the environment was revealed: 1,4-bis(2-ethylhexoxy)-1,4-dioxobutane-2-sulfonate (common name: dioctyl sulfosuccinate or DOSS). DOSS has also been used as a compound to track the distribution pattern of these dispersants (Campo et al., 2013). In order to reduce the buoyancy of the oil and increasing biodegradation, a total of 6.8 million liter of Corexit were applied to surfaced oil (58 %) and directly at the well (42 %) with varying application rates over the period of uncontrolled discharge of crude oil from the well (Ramseur, 2010; Kleindienst et al., 2015). Following the spatio-temporal distribution of the spilled crude oil, several sea- and air-going studies were conducted. These studies were able to confirm the presence of hydrocarbon plumes in the water column between approximately 1000 and 1200 m bsl. and a smaller plume between 50 and 500 m bsl. (Ryerson et al., 2012; Camilli et al., 2010). As the surface oil reached the shoreline from Texas to northern Florida (Nixon et al., 2016), the deep plume was detected drifting with the currents primarily in a southwestern direction from the well (Diercks et al., 2010, Kujawinski et al. 2011, Valentine et al., 2010). These data were evaluated by numerous studies calculating models by incorporating various parameters (oil and natural gas flow and properties, use of dispersing agents, biodegradation, temperature and currents) to ultimately predict hydrocarbon transport and plume formations (Lindo-Atichati et al., 2016; Socolofsky et al., 2015, Paris et al., 2012).

Reviewing the fate of hydrocarbons released in 2010, the portion of hydrocarbon oxidized and transformed by microbial processes is estimated to be the main sink (43 – 61 %), after deposition in the deep sea bed (2 – 15 %). The remaining fraction might have been deposited in coastal marshes or beaches (Fig. 1.1; Joye, 2015).

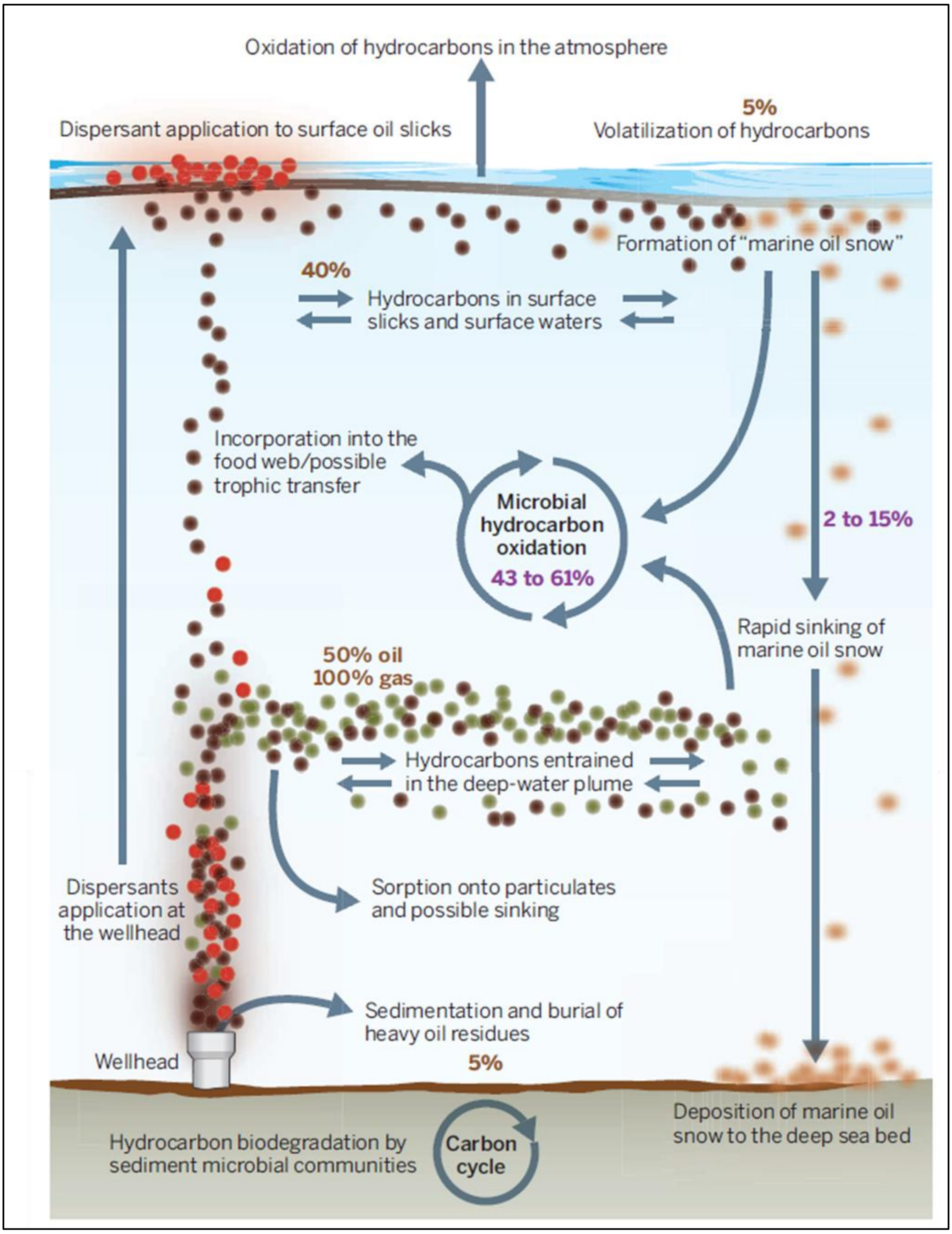


Fig. 1.1: Scheme of distribution and fate of crude oil and natural gases after the DWH oil spill. Adapted from Joye, 2015.

1.3. Crude oil Degradation by Bacteria

The fate and transport of discharged oil is determined by a complex interplay between hydrocarbon chemistry, the microbial food web, and ambient oceanographic processes including dispersion, dilution, dissolution, and advection by ocean currents, particle flocculation and aggregation, sedimentation, evaporation, along with biodegradation (Kostka et al., 2019). Microorganisms play a major role in the breakdown of the enormous amounts of hydrocarbons released into the deep sea. This chapter is taking a closer look at the source (crude oil) and the protagonists of microbial crude oil degradation (crude oil degrading bacteria).

- **Composition of Crude Oil**

Crude oil, like natural gases, is fossilized organic material in the Earth’s crust, which developed when layers of algae and zooplankton settled and were unable to be degraded under anoxic and stable temperature conditions. When getting buried, pressure and temperature increase and the organic material gets depleted of nitrate and sulfate by anaerobic bacterial metabolism. With further sedimentation, increase in pressure and in temperature, a mixture of organic matter (kerogen) is formed, which in turn forms different classes of nitrogen- and sulfur-depleted organic matter with different H/C-ratios (methane to asphaltenes) in time periods of several thousand years (Schobert, 2013). Crude oil is a complex mixture of more than 17,000 distinct chemical compounds and is categorized into four main classes: saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. The latter two are more polar than the saturated and aromatic hydrocarbons. The saturated hydrocarbons fraction in crude oil consists of varying proportions of carbon of C_1(methane) to C_{49} alkanes (C_nH_{2n+2}). The aromatic fraction consists of mono- (BTEX: benzene, toluene, ethylbenzene, xylene) and polycyclic aromatic compounds. Resins and asphaltenes are polar and high molecular weight compounds and consist of multiple aromatic ring structures. While resins are still soluble in crude oil, asphaltenes are colloidally suspended (Ollivier and Magot, 2005). Different oils, from separate sources, differ in their relative composition of these classes. Therefore crude oil is characterized by high hydrocarbon proportion (light oils) or higher polar fractions, i.e. resins and asphaltenes (heavy oils) (Head et al., 2006). The gravity of crude oil is measured and classified by the American Petroleum Institute (API) gravity, where light crude has an API above 31.1° and heavy crude oil below 22.3°. The crude oil from the Macondo well MC252, is characterized as a light crude oil with 40.0°, consisting of 74 % saturated, 16 % aromatic and 10 % polar hydrocarbons (Reddy et al., 2012). Degradation of saturated hydrocarbons is quantitatively the most important biodegradation step. A depletion of saturated and aromatic classes is usually a sign for biodegradation, as the polar classes are more resistant to biodegradation. Biodegradation of the crude oil can already start in the mostly anaerobic crude oil reservoirs (Head et al., 2006).

- **Degradation Pathways of Aliphatic and Aromatic Crude Oil Compounds**

Literature is available on the biodegradation of asphaltenes and resins (Hernández-López et al., 2015; Liao et al., 2009). However, as these compounds are not as susceptible to biodegradation as the saturated and aromatic hydrocarbons, they are not further discussed here. Alkanes, the most abundant class of crude oil, are chemically inert, due to the lack of functional groups. To exploit the energy and carbon source, organisms developed various metabolic strategies to activate the carbon chains for biodegradation that are outlined in the following (Ollivier and Magot, 2005). There is a variety of organisms able to perform this initial step: bacteria, yeasts, fungi and algae. Generally, pathways are distinguished by their usage of oxygen as electron acceptor into aerobic and anaerobic. Aerobic degradation of hydrocarbon (Fig. 1.2) is generally faster than anaerobic degradation. Within the phylum of bacteria the initial aerobic oxidative step is carried out by mono- and dioxygenases. Anaerobically there is a greater variety to initially oxidize hydrocarbon compounds, like the addition to fumarate, intra-aerobic hydroxylation, oxygen-independent hydroxylation and carboxylation (Ollivier and Magot, 2005, Kimes et al., 2014) (Tab. 1.1).

	Aerobic	**Anaerobic**
$C_2 - C_{10}$ **Short chain non methane alkanes**	• Non-heme iron monooxygenase similar to sMMO (C_2-C_9) • Copper-containing monooxygenase similar to pMMO (C_2-C_9) • Heme-iron monooxygenases (also refered as soluble cytochrome P450 (C_5-C_{12}) • Non-heme iron monooxygenase (*AlkB*-related) (C_3-C_{13} or C_{10}-C_{20})	• Fumarate addition
> C_{10} **Long chain alkanes**	• Heme-monooxygenase (P450 type) • [Fe^2]-monooxygenase • Non-heme iron monooxygenase (*AlkB*-related) (C_3-C_{13} or C_{10}-C_{20}) • Flavin-binding monooxygenase (*AlmA*) (C_{20}- C_{36}) • Thermophilic flavin-dependent monooxygenase (*LadA*) (C_{10}-C_{30})	• Fumarate addition • Carboxylation
Aromatic hydrocarbons	• [Fe]-Dioxygenase • [Fe^2]-Monooxygenase • [Flavin]-Monooxygenase	• Fumarate addition • Hydroxylation • Carboxylation

Tab. 1.1: Overview of aerobic and anaerobic mechanisms for hydrocarbon activation in bacteria. Adapted from Sierra-Garcia and Oliveira, 2013. MMO = methane monooxygenase, solubilized (s) and particulate (p) form. LadA = long chain alkane monooxygenase

Among the alkane activating enzymes of the aerobic pathways, the integral membrane non-heme iron monooxygenase (*AlkB*) is the most abundant and the best characterized. *AlkB* is found in bacteria able to degrade medium and long chain alkanes ($C_5 - C_{11}$ and > C_{12}). Aromatic structures are degraded aerobically via mono- or dihydroxylation of the aromatic ring and further the ring-cleavage with intra- or extradiol dioxygenases (I/ENDO). Alternative pathway is the CoA thioester pathway. Anaerobically hydroxylation pathways or again the addition to fumarate and further transformation to benzoyl-CoA are common pathways to degrade aromatic structures.

With further transformations, aliphatic and aromatic compounds are degraded via ß-oxidation and further metabolized via the tricarboxylic acid (TCA) cycle (Fig. 1.2).

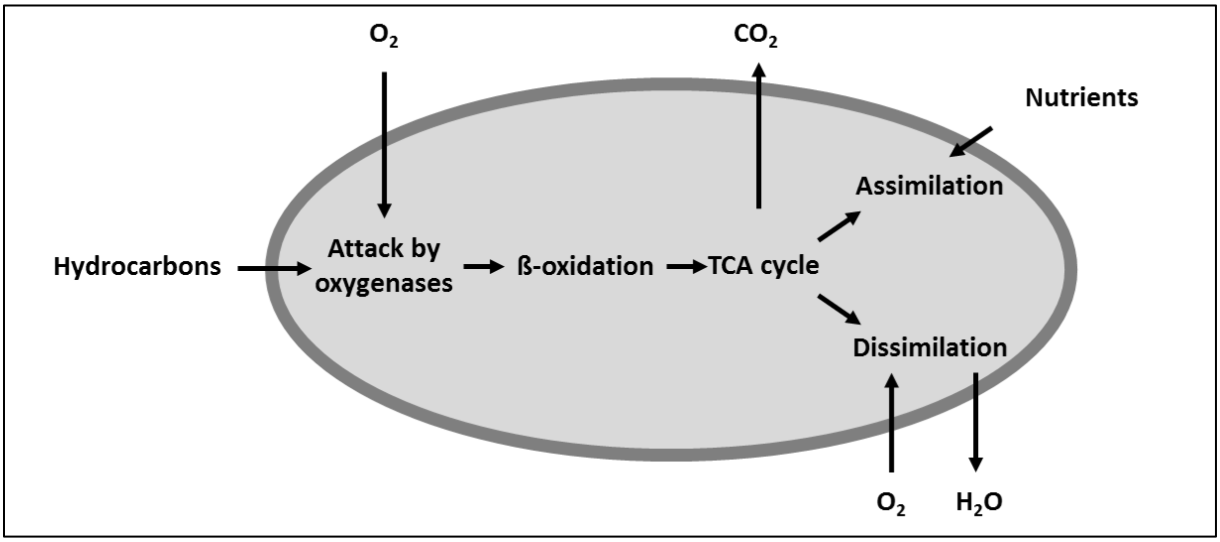


Fig. 1.2: Basic scheme of aerobic hydrocarbon degradation by bacteria. Adapted from Kleindienst and Joye, 2019.

In the phase of the uncontrolled flow of hydrocarbons and plume formation during the DWH, studies investigated the metabolic functional response to the input of hydrocarbons. 1600 genes related to aerobic and anaerobic hydrocarbon metabolism (alkane and PAH degradation) were found within the plume (Lu et al. 2012, Mason et al. 2012), at significant higher levels. Hereby, prominent aerobic *n*-alkane degrading genes (*alkB*) were found in moderate proportions ($< 10^{-4}$) of overall gene transcripts. PAH degrading genes were found to be less abundant, but concentrations of PAH in the plume were < 2 % of hydrocarbons.

Investigations of impacted surface sediment (0 – 1 cm) showed an increase in both aliphatic and aromatic degradation genes around the DWH well (Mason et al., 2014). Deeper sediment layers (1.5 – 3 cm) revealed a significant increase in anaerobic hydrocarbon degradation genes, such as *bssA*, benzoyl-CoA and *assA* (Kimes et al., 2013). Furthermore, the functional potential of an oil degrading community is bigger than those of a single species, which mostly have a limited substrate range. Additionally, some microbial representatives are able to produce extracellular polymeric substances (EPS) to increase bioavailability for the whole community (Gutierrez et al., 2013).

- **Hydrocarbon Degrading Taxa and the Succession of Degraders**

Publications on degradation of oil components date back to 1906 (Rahn, 1906). By 1950, Zobell (1950) already identified more than 100 species belonging to bacteria, yeasts, and molts, able to degrade hydrocarbons (Fig. 1.3). Isolates of several marine bacteria have been shown to exclusively degrade oil compounds, also called hydrocarbonoclastic bacteria (e.g. *Alcanivorax* spp., *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp.). Typically, a single organism is able to degrade just a limited range of hydrocarbons (Head et al, 2006).

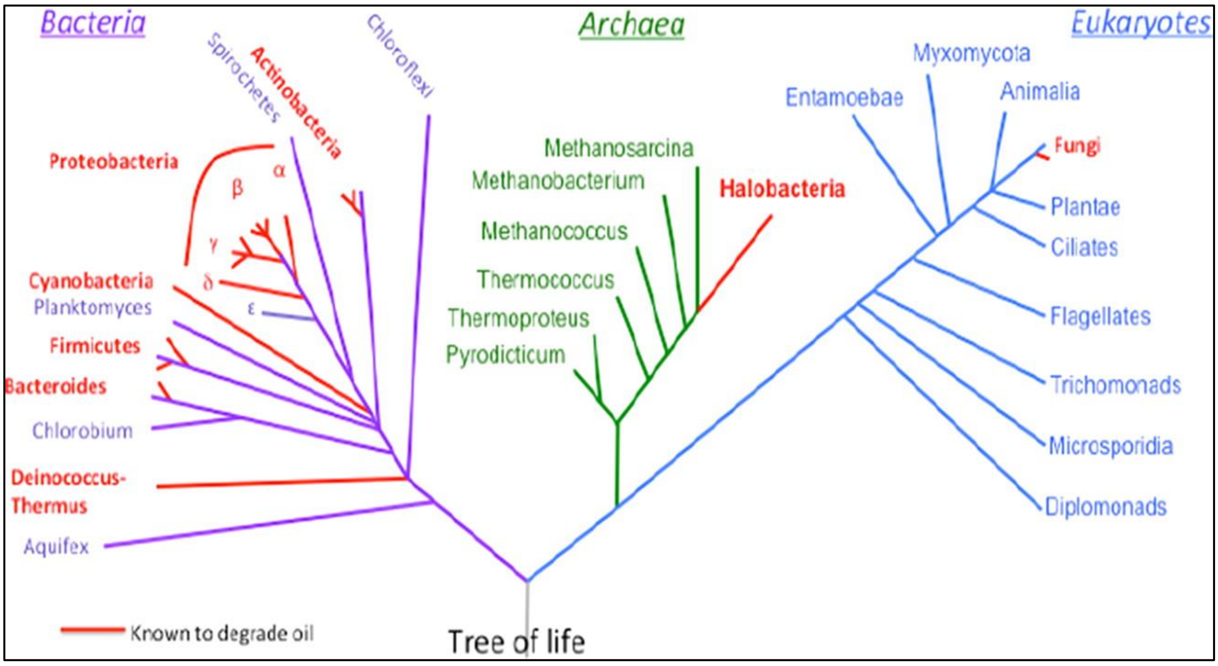


Fig. 1.3: *Tree of life indicating known hydrocarbon degrading phyla, highlighted in red. Adapted from Hazen et al. 2016.*

The plume, which formed after the DWH explosion and during the uncontrolled flow of hydrocarbons between a depth of 1000 and 1200 m, consisted of alkanes, BTEX and PAHs (Reddy et al, 2012). In consequence, overall functional diversity and microbial biomass within the plume increased from 2.7×10^4 cells cm^{-3} outside the plume to 5.5×10^4 cells cm^{-3} within the plume (Lu et al., 2012). However, as cell numbers increased, bacterial diversity dropped drastically down to one species dominating 90 % of all reads, while accounting for only 3 % outside the plume (Hazen et al., 2010, Mason et al., 2012). The plume bacterial community differed significantly from pre-spill and non-plume samples ($p < 0.01$; Dubinsky et al., 2013).

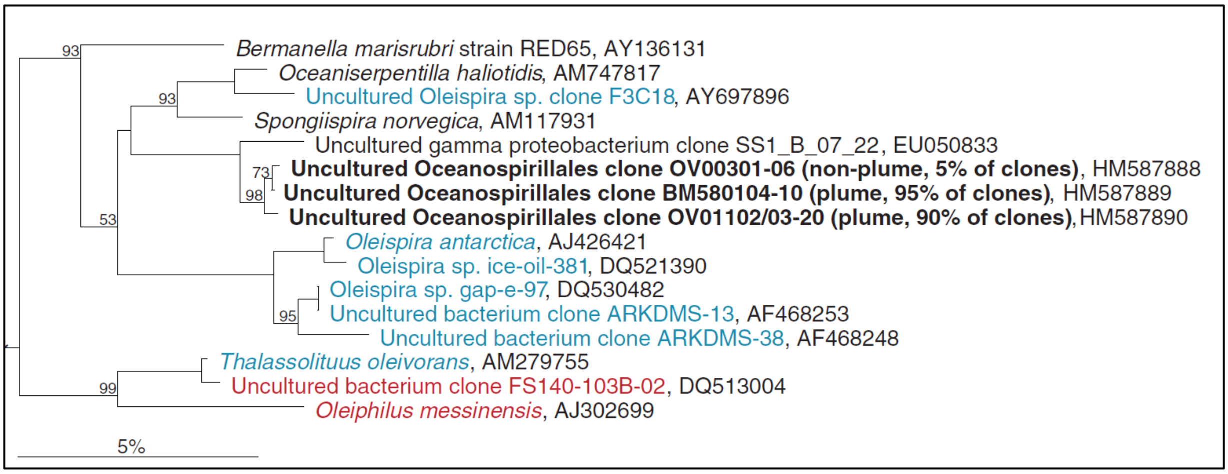


Fig. 1.4: Phylogenetic tree showing the relationship of the plume dominant bacteria, all belonging to the Gammaproteobacteria. Known psychrophilic hydrocarbon degrading species (blue) and known hydrocarbon degraders from not psychrophilic environments (red) are indicated. Adapted from Hazen et al., 2010.

The responding genera (Fig. 1.4) belonged nearly exclusively to the Gammaproteobacteria and therein to just four families: Colwelliaceae, Methylococcaceae, Oceanospirillaceae, and Piscirickettsiaceae (Rivers et al, 2013). Further Alteromonadales, Deltaproteobacteria, Pseudomonadales, and SAR86 were present, but not to the same extent (Mason et al., 2012).

Sea-going research (Hazen et al., 2010, Redmond and Valentine et al., 2012, Dubinsky et al., 2013) found a change in the dominant species over time (Fig. 1.5). In the period of uncontrolled oil flow (April 25th – June 4th) Oceanospirillaceae dominated the plume, with underlying elevated abundance of *Colwellia*, *Cycloclasticus*, *Pseudomonas*, *Pseudoalteromonas*, and *Thalassomonas* (Mason et al, 2012, Dubinsky et al., 2013). The following bloom of mainly *Cycloclasticus* and *Colwellia* (Valentine et al.,2010; Redmond and Valentine, 2012) as well as *Pseudoalteromonas*, *Methylomonas*, and *Thalassomonas* took place during partial hydrocarbon capture (June 5th – July 14th). After the well was capped (July 15th onward), Flavobacteria (*Tenacibaculum* and *Polaribacter*), Alteromonadaceae, and Rhodobacteraceae dominated the community. It is believed that the succession of dominant bacterial species bloomed as long as their preferred hydrocarbon source was available (Dubinsky et al., 2013). Oceanospirillaceae are assumed to have high growth rates on short chain alkanes (preferably butane and propane, Valentine et al., 2010 and 2012). *Colwellia* grew slower due to their broad substrate range, including gaseous hydrocarbons and PAHs (Redmond and Valentine, 2012) and might have dominated after depletion of short chain hydrocarbons in the plume. The detected genus *Cycloclasticus* is known to degrade cyclic hydrocarbons (Gutierrez et al., 2013). Methane was the most abundant hydrocarbon released, which led to blooms of methane oxidizers, like *Methylomonas* (Methylococcaceae) in late June and August. The taxa, which became dominant after the well was shut-in, are known to degrade high-molecular-weight hydrocarbons and dissolved organic matter (DeLong et al., 1993, Kirchman, 2002).

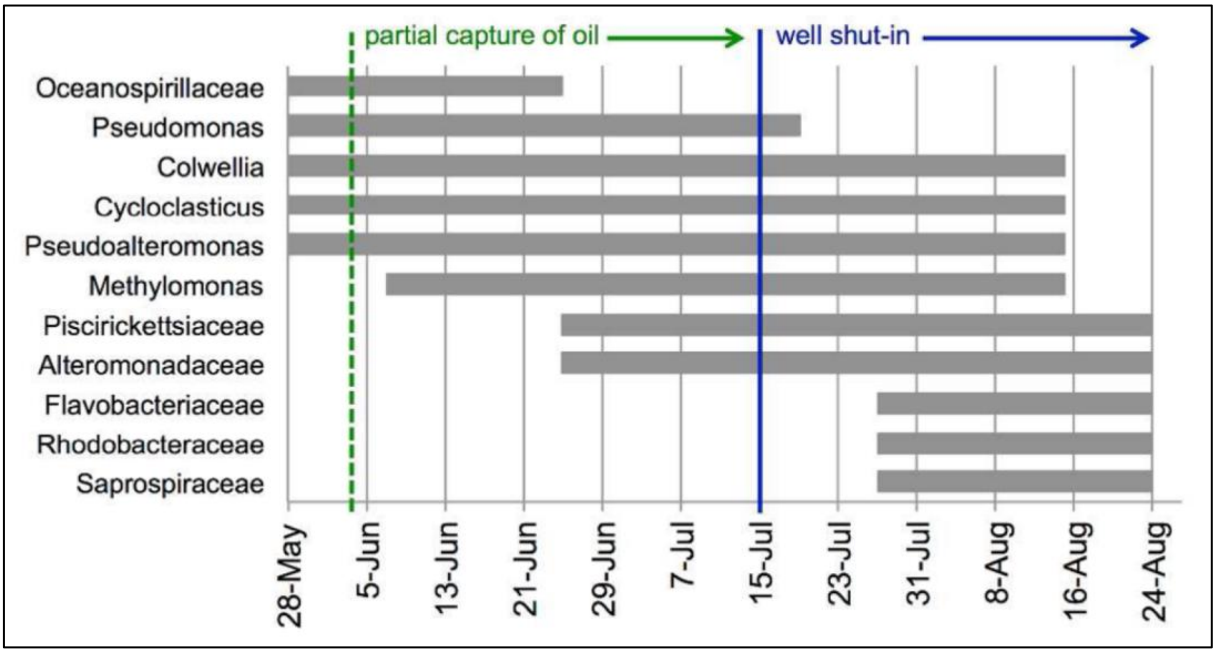


Fig. 1.5: Periods of dominance of taxa within the plume. Bars are the periods where relative abundance was enriched over background in any sample (> 2-fold mean non-plume intensity). Adapted from Dubinsky et al., 2013.

In the upper sediments close to the well, elevated PAH concentrations were detected up to 500-fold compared to unaffected sites (Kimes, 2014). This induced an increased abundance of an uncultured Gammaproteobacterium and a *Colwellia* species, and an increase in metabolic marker genes for degradation of aliphatic and simple aromatic hydrocarbons (Mason et al., 2014). A study investigating upper sediment of two sites 2 and 6 km from the well in May 2011 revealed an increased diversity and species abundance, which are normally associated with natural seep sites (*Methylococcus*, *Methylobacter*, *Actinobacteria*, *Firmicutes*, and *Chlorofexi*, Liu and Liu, 2013). One year later, *n*-alkane abundances were relatively enhanced in comparison to PAH abundances (10 - 1000 times) (Liu et al., 2012) in the sediment, with corresponding functional genes for the degradation of PAHs found (Kimes et al., 2013). This suggested that the degradation of PAH was faster than the degradation of alkanes in the sediment. A later study (Bagby et al., 2016) in the close proximity of the well (1 – 6 km) detected oil components in the upper sediment four years after the spill. They found signs for a degradation succession, but also persistent alkanes and PAH compounds suspended in larger droplets buried in the sediment.

Liu and Liu (2013) detected the presence of *Rhodococcus* (Actinobacteria) in the investigated sediments. *Rhodococcus* spp. have been reported to effectively degrade *n*-alkanes and aromatic hydrocarbons (Sorkhoh et al., 1990), and demonstrated a strong bioremediation potential (De Carvalho & Da Fonseca, 2005). This potential probably led to *Rhodococcus* sp. becoming dominant in isolation experiments (Sun, 2014), using deep sea sediment from the GoM as inoculum. We were able to isolate a *Cobetia* (Oceanospirillales, Halomonadceae) strain from a high-pressure incubation of deep sea sediment in this study. *Cobetia* has been isolated from marine waters near the coast. *Cobetia* is known to produce biosurfactants, and to degrade polyaromatic compounds (like phenanthrene). While the closely related genus *Halomonas* was reported as a prominent hydrocarbon degrader in surface waters after the DWH incident, C*obetia* was not detected (Ferguson et al., 2017).

- **Toxic Effect of Crude Oil and Dispersant to Marine Organisms**

Crude oil and dispersant toxicity studies after the DWH incident have been performed on higher trophic levels, like small fish and shrimp, which generally are impacted by the exposure. The crude oil compounds responsible for the toxic effect in aquatic organisms are short-chain alkanes and two- or three-ring PAHs. Therefore, toxicity decreases parallel to the weathering of crude oil (Fuller et al., 2004). Adding dispersant to the crude oil caused an increase in toxicity for marine rotifer (*Brachionus manjavacas*, Rico-Martínez et al., 2013), mysid shrimp (*Americamysis bahia*) and inland silverside fish (*Menidia beryllina*) (Hemmer et al., 2011). Dispersed oils were more toxic than the crude oil or dispersing agent alone. Enhanced toxicity by the dispersed oil might be induced by an increase in the dissolution rate through an enlarged surface area of the crude oil.

Generally, toxicity tests on microorganisms are rare (Blum and Speece, 1991), but a standardized toxicity test on marine fluorescent bacterium *Vibrio fischeri* exists. Toxicological tests with dispersed crude oil have been performed on different strains and led to inconclusive results, with growth enhancement and retardation (Garcia et al., 2001, Fuller et al., 2004, Overholt et al., 2016). As discussed above, natural oil spill sites are frequently occurring along the northern GoM shelf. Subsequently the marine pelagic bacterial community is believed to have adapted to the input of crude oil, and analysis of expressed genes compared to present genes indicated that most taxa were unaffected, while only 5 % of the taxa were inhibited by the input of crude oil (Rivers et al., 2013). In general, crude oil consists of numerous different carbon sources that would favor generalists in the early succession, as well as toxic compounds that would preferentially select against specialists. After the disturbance event (here: hydrocarbon input), communities are assumed to be characterized by a narrower set of more specialized functions (Rodriguez-R et al., 2015).

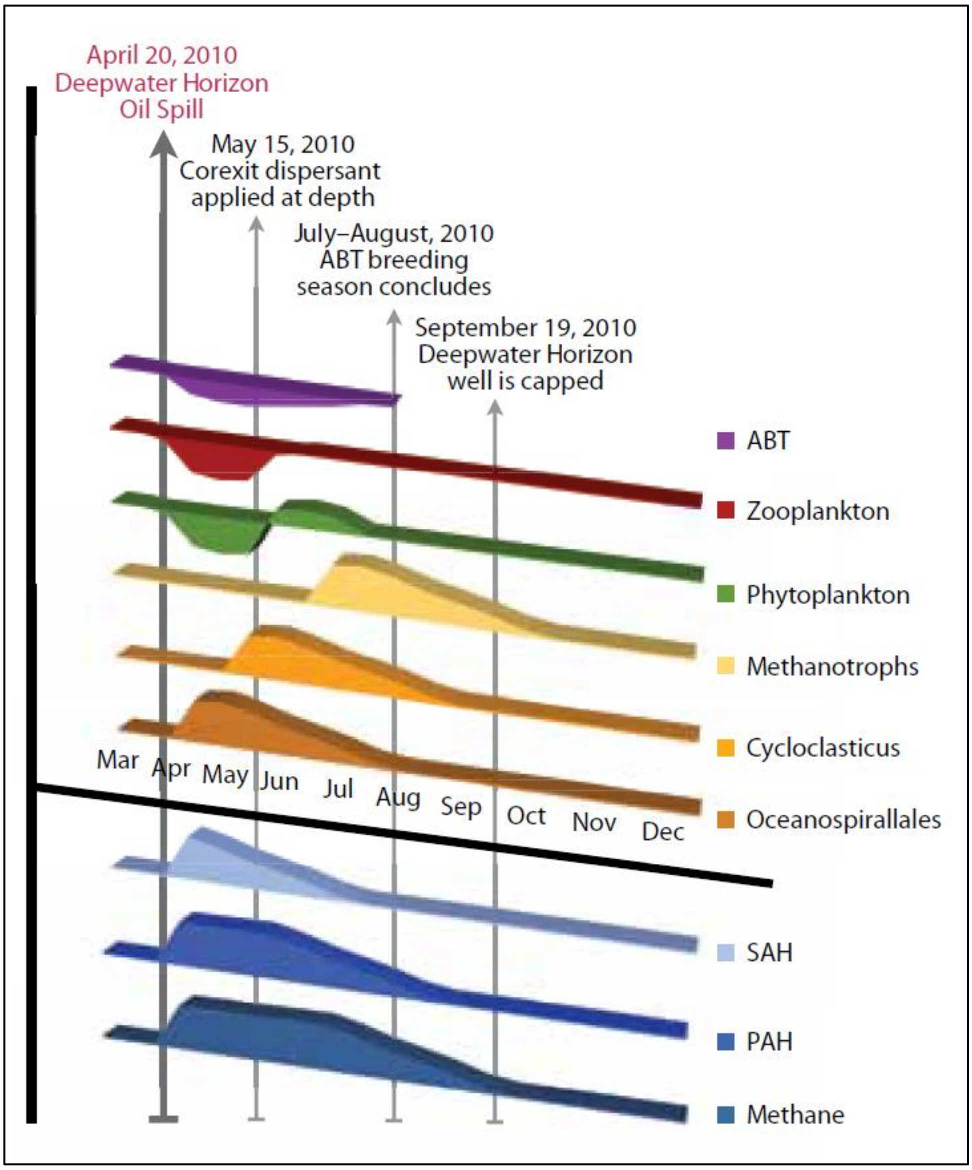


Fig. 1.6: Time line of oil spill driven changes in the oil compound classes (ABT = atlantic bluefin tuna, SAH = simple aliphatic hydrocarbons; PAH = polycyclic aromatic hydrocarbons) affecting relative organism classes' abundances. Adapted from Abbriano et al. (2011).

1.4. The Influence of Pressure on Bacterial Growth and Activity

The interest in the influence of hydrostatic pressure on microorganisms has a long history, dating back to 1884 (Certes, 1884). Zobell and Oppenheimer (1950) investigated the effect of elevated pressure on barophilic marine organisms, and already indicated that microbial research might miss something when it tries to investigate deep sea ecological processes without taking pressure as a factor into consideration. Life can be found in the pressure range from -2 MPa (xylem of plants) to the subsurface of the deepest oceans (> 110 MPa) (Bartlett, 2002). Some *Colwellia* species (*Colwellia* MT41, DeLong et al., 1997) have been described with a growth optimum of 103 MPa. Meanwhile the cultivation of microorganisms under high pressure has been realized (Mikucki et al., 2003, Orcutt et al., 2011) and general effects on microbial life has been investigated and reviewed extensively (Bartlett, 2002). Studies on *Escherichia coli* showed that pressure inhibits cell functions differently. While the motility is already inhibited at 10 MPa (Meganathan and Marquis, 1973), translation and transcription are still functional at 60 and 77 MPa, respectively (Yayanos and Pollard, 1969). Pressure could induce heat- and cold-shock proteins in *E. coli* simultaneously (Bartlett, 2002). Further, cell functions reported to be affected by pressure are gene expression (Welch and Bartlett, 1998, Scoma et al., 2016a and 2016b), ratios of membrane lipids (Allen et al., 1999, Grossi et al., 2010), specific membrane proteins (Chi and Bartlett, 1995), decrease in DNA content (Chastain and Yayanos, 1991), increased DNA supercoiling (Tang et al., 1998), and potential filament formation outside of the pressure optimum (Jannasch, 1987). In addition, an adaptation to elevated pressure in an initially piezo-sensitive *E. coli* strain was observed (Marietou et al., 2015). How metabolic processes are affected by pressure is rarely investigated. For example, a methanogenic strain (*Methanopyrus kandleri*) was isolated at 40 MPa and 122 °C and shown to drastically alter carbon fractionation when incubated at different pressures (Takai et al., 2008). There is a limited number of publications available focusing on crude oil degradation under elevated pressure (reviewed by Scoma et al., 2016c). This, however, is crucial in explaining microbial ecological processes in elevated pressure environments like the deep sea. The information obtained from hydrocarbon degradation experiments under elevated pressure with single strains revealed different strategies to adapt to elevated pressure and varying degrees of piezo-tolerance. Single organisms were able to grow and degrade crude oil components under pressures up to 35 MPa (Grossi et al. 2010, Hackbusch et al., 2019). Other, obviously piezo-sensitive hydrocarbon degraders, were reduced in growth and degradation efficiency (Scoma et al., 2016a and 2016b, Schedler et al., 2014).

Reviewing the behavior of bacterial communities under elevated pressure (50 MPa), Schwarz et al. (1974, 1975) already indicated reduced degradation rates of alkanes by a deep sea community, mixed from deep sea sediments (4940 m depth). A more recent study on the upper GoM sediment under oxic *in situ* conditions (Nguyen et al., 2018) found, among other influencing factors, a 4 % decrease in total *n*-alkane degradation per 1 MPa increase. Marietou et al. (2018) showed in another *ex situ* study on pelagic (1070 m depth) GoM seawater that *in situ* pressure and temperature reduced the growth and metabolic potential of hydrocarbon degraders. They also pointed out that a synergistic effect of pressure and temperature influenced community dynamics.

1.5. Scope of This Thesis

- **Gulf of Mexico Research Initiative (GoMRI) and Center for Integrated Modeling and Analysis of Gulf Ecosystems (C-Image)**

The Gulf of Mexico Research Initiative (GoMRI) was founded by BP (former British Petroleum) in May 2010 after the DWH. BP was leasing the DWH platform and committed, after the DWH incident, a grand of 500 million US Dollar over a 10-year-period to an independent research program, primarily in the US Gulf States. Aim of GoMRI is to investigate the impacts of crude oil and dispersant on the ecosystem of the Gulf, human health, and the coastal states, with the ultimate goal to improve response efforts after oil spills (http://gulfresearchinitiative.org/about-gomri/gri-history/, accessed March 2019). One of the funded consortia starting in 2011 was C-Image (Center for Integrated Modeling and Analysis of Gulf Ecosystems), consisting of 19 international institutions from six countries. To advance the understanding of marine blowouts, C-Image II (second funding period) was addressing six tasks (Task 1: Near-Field & Far-Field Modeling; Task 2: High-Pressure, Low Temperature Environments; Task 3: The Distribution, Fate, Transport and Impacts of Oil Deposition Resulting from MOSSFA Processes During DWH and the IXTOC-I Discharges (comparison to an earlier oil spill in the GoM); Task 4: Hydrocarbon Impacts on Plankton, Invertebrates, Fishes and Marine Mammals; Task 5: Toxicology Studies; Task 6: Detailed and Spatially Resolved Ecosystem Modeling). This present study is part of the Task 2: High-Pressure, Low

Temperature Environments by investigating hydrocarbon degrading bacteria and bacterial communities under deep sea environmental conditions.

- **Research Objective of This Thesis**

A variety of literature, covering laboratory and field research is available on hydrocarbon degradation (Hazen et al., 2015). Most of the biodegradation studies have been performed *ex situ* under conditions resembling marine surface waters. Relatively few studies were set to mimic deep sea conditions with low temperature and elevated hydrostatic pressure. This very environment has been severely impacted by the DWH explosion. Generally, industrial oil production will continue to exploit oil fields further off shore (Fig. 1.7, Kennicutt, 2017, Maribus, 2014) in ultra-deep waters (> 1500 m depth). As biodegradation is of major importance in ultimately removing crude oil components from the environment, the insight in the persistence of different oil fractions is vital to manage oil spill responses and recovery. The knowledge gap of deep sea bacterial communities' response to massive hydrocarbon input in an otherwise oligotrophic environment is critical to effectively parameterize oil plume models (Kostka et al., 2019).

The response of the bacterial community to the DWH hydrocarbon input was well investigated in the field (Hazen et al., 2010, Redmond and Valentine et al., 2012, Mason et al., 2012), and in the lab under surface conditions (Gutierrez et al., 2013, Mason et al., 2014, Yergeau et al., 2015). Just a handful of laboratory studies were able to implement pressure as an oceanographic factor in investigations on crude oil degradation. Those (Nguyen et al., 2018, Marietou et al., 2018) detected a reduction of metabolic activity in the hydrocarbon degrading community at elevated pressure.

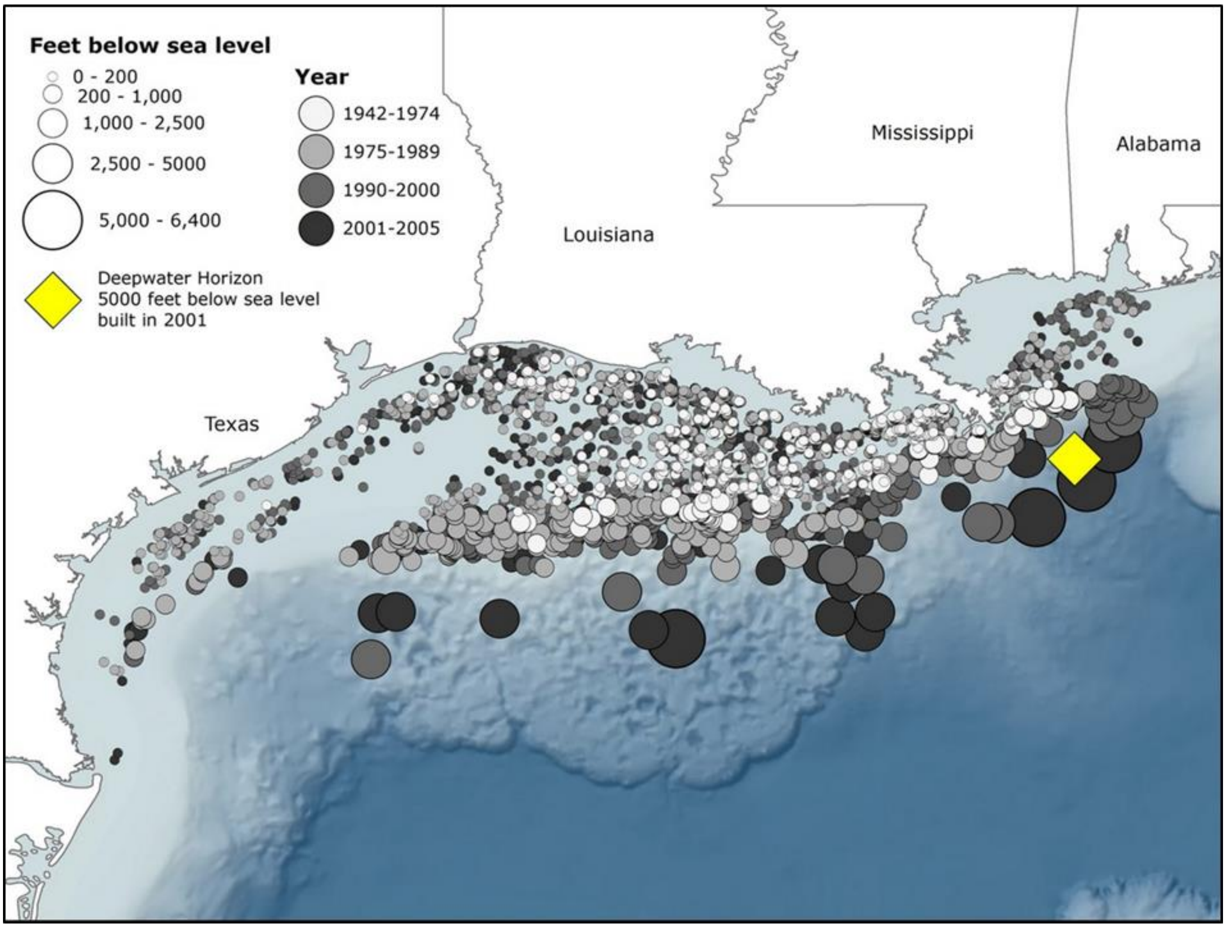


Fig. 1.7: History of marine drilling in the northwestern Gulf of Mexico. Color-coding for year of establishment, size-coding for depth below sea level (1 feet = 0.3048 meter). Yellow diamond shape for DWH position. Adapted from Bos, Carole "Location of Oil Rigs in the Gulf of Mexico" AwesomeStories.com. Feb 24, 2016. Jun 11, 2019.

Changes in the composition of the bacterial community are of major importance to evaluate the influence of and resilience towards factors like crude oil, hydrostatic pressure and the addition of dispersant. The research in this thesis choose different approaches to enhance the knowledge about these influencing factors on the microbial life of the deep sea:

The initial approach is a **single strain approach** with *Rhodococcus* sp. PC20 strain, which was isolated from the GoM deep sea sediment. The aim is to investigate effects of pressure and dispersant on a single crude oil degrading strain. These species-specific responses are quantified and compared via culture dependent methods.

Ecological considerations, such as biotic interactions and resilience of the entire deep sea community, are assessed in a **community approach**. Deep sea sediment is incubated at different combinations of the environmental factors pressure, dispersant and crude oil, similar to the conditions of the DWH oil spill. Hereby, the relative abundance of the present taxa is investigated with next generation sequencing (NGS) methods. Changes in the bacterial community should help to identify taxa specifically responding to environmental factors and to statistically evaluate each environmental factors importance. NGS is a powerful and quickly developing tool, which gives large quantities of information. However, the pure (relative) presence of a known hydrocarbon degrading taxon, does not automatically imply its activity.

Therefore the final approach is closely linked to the community approach. Following high pressure incubations of deep sea sediment, an **isolation approach** is conducted, with the goal to isolate hydrocarbon degraders under elevated pressure conditions. Ideally, to isolate those strains, which become dominant in the community approach experiments. After isolation and identification of isolated strains, experiments similar to those of the single strain approach are conducted. These experiments are carried out with culture dependent methods.

2. Material & Methods

2.1. Experimental Procedures

2.1.1. High Pressure Setup

The technical equipment for high pressure applications were constructed and maintained by the company Technik Service Andreas Meyer (Lindau, Germany). The reactors (n = 10) were designed and applied in experiments by Martina Schedler and Ana-Gabriela Valadarez-Juarez (Schedler et al., 2014, Schedler, 2017). A scheme of the reactor setup is shown in Fig. 2.1. Each reactor consists of a stainless-steel tube (diameter / height: 41 mm / 132 – 151 mm) and a bronze lid at bottom and top, each furnished with an inside thread and a total volume between 160 – 170 mL. The upper bronze lid has a maximum of two inlets. One for building up pressure and releasing it, equipped with a valve, a manometer and a safety reflux connection to attach the connection to the nitrogen gas reservoir. Some of the reactors (n = 8) have the option to optically measure the oxygen concentrations in the headspace of the reactor. For this, the inlet is equipped with a pressure tight glass fiber screw joint. The tip of the glass fiber, which is facing inwards, has a sensor spot with oxygen sensitive coating (SP-PSt3-NAU, PreSens, Regensburg, Germany) glued to it. This enables an oxygen measurement after a two-point calibration (0 / 100 %), also at elevated pressure. A Fibox3 system (PreSens, Regensburg, Germany) was used to convert the optical signal into a concentration of oxygen. The reactors were pressurized with nitrogen gas (N_2) up to 15 MPa. Nitrogen gas was used for pressurization, as the increase in oxygen concentration with pressurization with oxygen (O_2) or ambient air can become toxic to microorganisms. Reactors were cleaned after and before use with ethanol and were covered with aluminum foil when not in use.

- ***Pressurization Procedure***

To set up the high pressure incubation, medium, inoculum and substrates were added to the autoclaved reaction vials aseptically and sealed with aluminium foil. Subsequently, the vials were inserted into the high pressure reactors and the aluminium foil was punctured with a sterile needle to ensure gas exchange in the reactor. The bronze lid was closed after equilibration with ambient air. If methane (Methan 2.5, > 99.5 % purity, Linde, Pullach, Germany) was added to the reaction, the safety reflux bolted connection (Hy-Lok D Vertriebs GmbH, Oyten, Germany) was attached to the methane gas bottle and 0.1 MPa of methane was adjusted at the gas bottles' safety valve with the needle valve (Hy-Lok D Vertriebs GmbH, Oyten, Germany) closed. By slowly opening the needle valve, a constant increase in pressure inside the reactor was assured. The same procedure was repeated when a subsequent pressurization with nitrogen gas (Stickstoff 2.8, >99.8 % purity, Linde, Pullach, Germany) was needed to simulate deep sea hydrostatic pressure. After pressurization, the needle valve was closed and the pressure between bottle and safety reflux bolted connection was released before detachment. Safety glasses had to be worn during the whole procedure. Further, it was of great importance during the depressurization procedure, that an open window ensured sufficient oxygen availability in the lab.

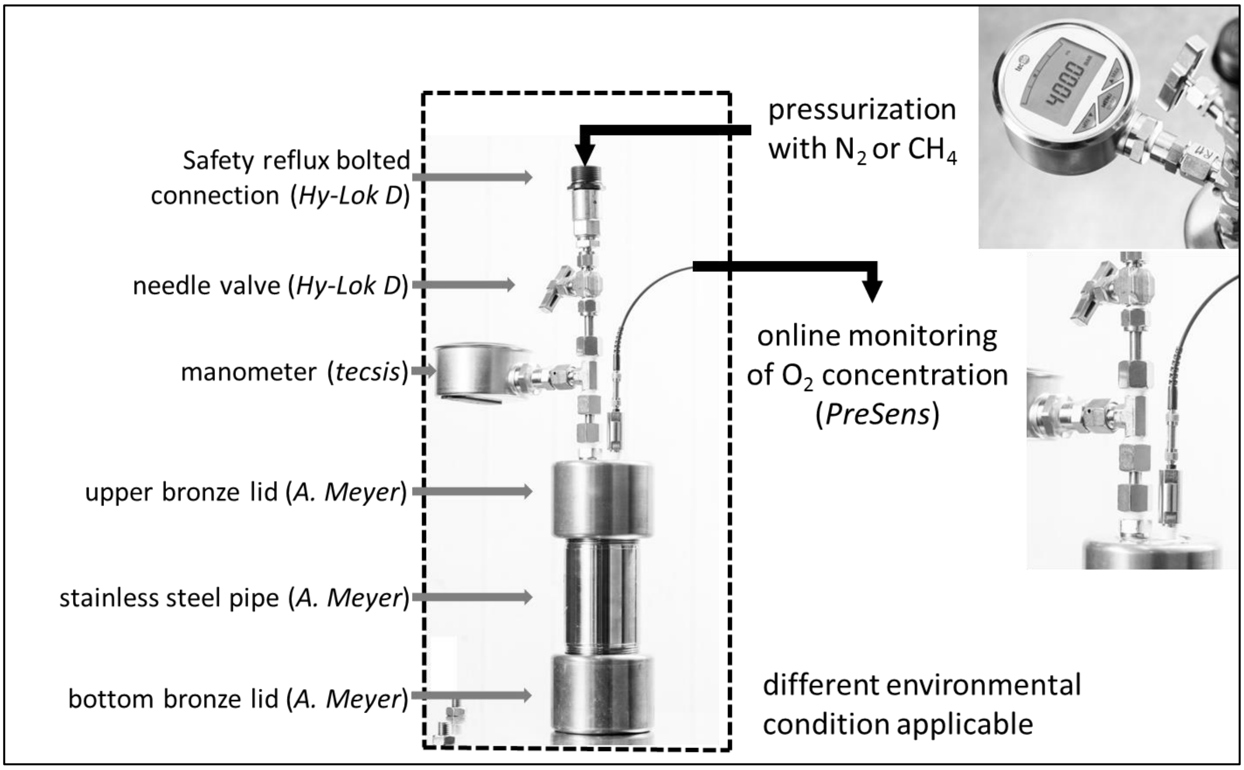


Fig. 2.1: Setup and technical features of the high pressure reactor, with compounds purchased from the companies hy-lok D Vertriebs GmbH (Oyten, Germany), tecsis GmbH (Offenbach am Main, Germany), PreSens (Regensburg, Germany) and A.Meyer (Lindau, Germany).

2.1.2. Substrates

A variety of substrates was used in the experiments: complex standard substrate for bacterial growth (Lysogeny Broth (LB)), single hydrocarbon compounds (*n*-alkanes, aromatic compounds), and crude oil. Some experiments were conducted by adding a dispersing agent, Corexit EC9500A, as it was applied during clean-up measures after the DWH oil spill. The following paragraphs include the type of substrate, handling during and storage in between experiments.

▪ *Crude Oil*

Louisiana sweet crude oil (request ID 10384; Surrogate SO-20111116-MPDF-003 A0066F OL-Oil) was obtained from the Knox Storage Archive Facility (Fort Collins, Colorado, USA). This oil is a surrogate oil from the Marlin platform of the Dorado field, which is located 37 km northeast of the DWH platform. Surrogate means, this oil is chemically and toxicologically similar to those of the Macondo field oil (Schedler, 2017). The aliquoted crude oil was autoclaved and stored at 4 °C until further use. In the experiments, crude oil was added in different amounts, always in relation to the amount of medium applied (0.025 – 2 % v/v). The applied amount was chosen from similar *ex situ* experiments (Schedler et al., 2014, Techtmann et al., 2017, Kleindienst et al., 2015, Prince et al., 2016). Due to the physiological properties of crude oil, the oil was added to the reaction with a glass syringe.

▪ *Dispersant*

The dispersing agent, which was applied during the DWH oil spill was ordered from the company Nalco (Naperville, Illinois, USA). The aliquoted dispersant was autoclaved and stored at 4°C until further use. Corexit®EC9500A was used in experiments in relation to the oil added. These dispersant to oil ratios (DOR) ranged from 1:20 to 1:1000. The dispersant was added to the incubation medium with small pipets after the addition of crude oil.

▪ n-*Alkanes and Aromatic Compounds*

Different single hydrocarbons (*n*-hexane, *n*-heptane, *n*-octane, *n*-hexadecane, benzene, naphthalene) were tested as substrates for *Rhodococcus* PC20 and Cobetia C6 / C8. For comparability to other substrates, their concentrations were normalized to the carbon content. These concentrations differed by the experiments 16 / 160 mmol of carbon per liter (Tab. 2.1).

	Experiment J1 (Fig. 3.5)			Experiment J2 (Fig. 3.6)			
	***n*-hexadecane**	**benzene**	**naphthalene**	***n*-hexane**	***n*-heptane**	***n*-decane**	***n*-hexadecane**
#C-atoms	16	6	10	6	7	10	16
Conc. [mmol C / L]	**16**	**16**	**16**	**160**	**160**	**160**	**160**
Conc. [mmol / L]	1.0	2.7	1.6	26.7	22.9	16.0	10.0
g / L	0.227	0.211	0.205	2.302	2.276	2.278	2.264

Tab. 2.1: Concentrations of single hydrocarbon compounds used in Rhodococcus *PC20 experiments.*

- ***Lysogeny Broth (LB)***

As a reference for a different complex medium Lysogeny Broth (LB) was used to compare the growth of single strains with crude oil. LB medium consisted of 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl at pH of 7.0 ± 0.2. 25 g of the LB mixture (Luria-Miller; C. Roth, Karlsruhe, Germany) were dissolved in 1 liter of medium. For the preparation of solid agar plates 15.0 g of agar-agar (bacteriologically, C. Roth, Karlsruhe, Germany) were added to the mixture, autoclaved and poured into sterile petri dishes under the clean bench.

2.1.3. Growth Media

Different culture media were tested and used in the conducted experiments. Also different media were used for incubation (MM2, ASW, FSW) and two different types of solid agar plates (LB and MA) for quantification of colonies were used.

- ***Mineral Minimal Medium (MM2)***

Mineral minimal medium (MM2) is a minimal medium with high concentration of phosphorus (28.1 mM) and ammonium (15.1 mM). It consist of 2.6 g Na_2HPO_4, 1.33 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_20$ filled up to 1 liter. Additionally 3 % w/w (30 g) NaCl were added to adjust for seawater salinity conditions. After autoclaving 1 % of vitamin solution and 10 mL of trace element solution (both Tab. 2.2) were added aseptically. Both solutions were adapted from DSMZ medium Nr. 141. For producing the trace element solution, nitrilotriacetic acid was dissolved and then the medium was adjusted to pH 6.5 with KOH, afterwards the minerals were added. The final pH had to be adjusted to 7.0 (with KOH). Both solutions were sterile filtered after preparation and again before use (0.2 µm CA / PES, Corning 1L, Sigma Aldrich). Sodium tungstate was not included in the recipe, due to no availability and long shipping times (> 3 months).

Trace element solution	
Compound	**Amount**
Nitrilotriacetic acid	1.50 g
$MgSO_4$ x 7 H_2O	3.00 g
$MnSO_4$ x H_2O	0.50 g
NaCl	1.00 g
$FeSO_4$ x 7 H_2O	0.10 g
$CoSO_4$ x 7 H_2O	0.18 g
$CaCl_2$ x 2 H_2O	0.10 g
$ZnSO_4$ x 7 H_2O	0.18 g
$CuSO_4$ x 5 H_2O	0.01 g
$KAl(SO_4)_2$ x 12 H_2O	0.02 g
H_3BO_3	0.01 g
Na_2MoO_4 x 2 H_2O	0.01 g
$NiCl_2$ x 6 H_2O	0.03 g
Na_2SeO_3 x 5 H_2O	0.30 mg
~~Na_2WO_4 x 2 H_2O~~	~~0.40 mg~~
Distilled water	1000.00 mL

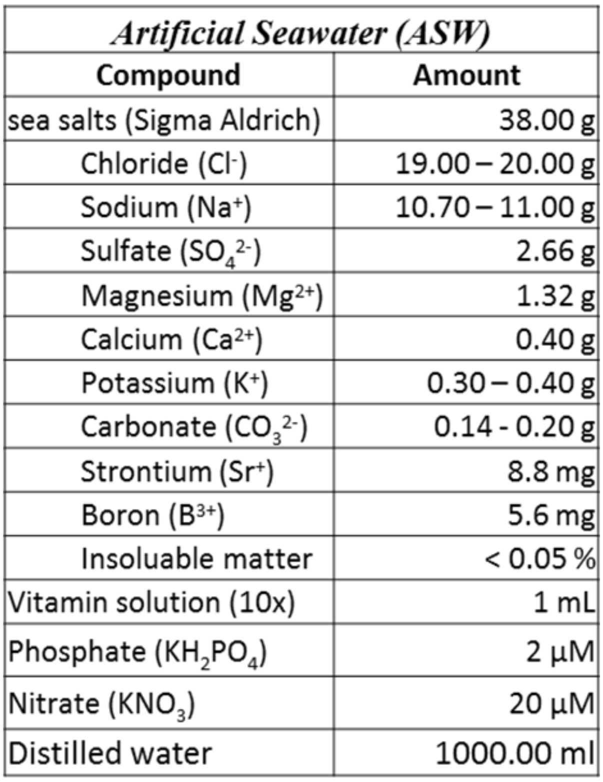
Vitamin mixture solution (10x)	
Compound	**Amount**
Biotin	2.00 mg
Folic acid	2.00 mg
Pyridoxine-HCl	10.00 mg
Thiamine-HCl x 2 H2O	5.00 mg
Riboflavin	5.00 mg
Nicotinic acid	5.00 mg
D-Ca-pantothenate	5.00 mg
Vitamin B12	0.10 mg
p-Aminobenzoic acid	5.00 mg
Lipoic acid	5.00 mg
Distilled water	100.00 mL

Tab. 2.2: Trace element solution (DSMZ 141) and 10 fold concentrated vitamin mixture (DSMZ 141) composition. Sodium tungstate (Na_2WO_4 x 2 $H_2$0) was not added to the medium.

- ***Artificial Seawater (ASW)***

Artificial Seawater (ASW) was prepared to simulated seawater conditions as closely as possible. The composition of the ASW is shown in Tab. 2.3. All compounds were mixed in a sterile way under the clean bench and were subsequently sterile filtered (0.2 µm CA / PES, Corning 1L, Sigma Aldrich, Karlsruhe, Germany).

Nitrate and phosphorus contained in inorganic salts or organic compounds were added in the amounts (20 / 2 µM), which were measured by the R/V WeatherBird II for the deep sea stations.



Artificial Seawater (ASW)	
Compound	**Amount**
sea salts (Sigma Aldrich)	38.00 g
Chloride (Cl^-)	19.00 – 20.00 g
Sodium (Na^+)	10.70 – 11.00 g
Sulfate (SO_4^{2-})	2.66 g
Magnesium (Mg^{2+})	1.32 g
Calcium (Ca^{2+})	0.40 g
Potassium (K^+)	0.30 – 0.40 g
Carbonate (CO_3^{2-})	0.14 - 0.20 g
Strontium (Sr^+)	8.8 mg
Boron (B^{3+})	5.6 mg
Insoluable matter	< 0.05 %
Vitamin solution (10x)	1 mL
Phosphate (KH_2PO_4)	2 µM
Nitrate (KNO_3)	20 µM
Distilled water	1000.00 ml

Tab. 2.3: Composition of the Artificial Seawater with the sea salts mixture (Sigma Aldrich). Vitamin mixture and trace element were added as described in the text.

- ***Filtered Seawater (FSW)***

Samples of seawater from the deep sea of the GoM were sterile filtered and compared to the artificially produced seawater (ASW). Deep sea bottom water samples from stations DWH01, DSH10, and SW1 (each approx. 2 L) were collected and shipped by the R/V WeatherBird II (WB0816) crew in August 2016 and stored at 4 °C. Samples were further stored at 4 °C until being sterile filtered (0.2 µm CA / PES, Corning 1L, Sigma Aldrich) and used in the experiments.

- ***Marine Agar (MA)***

For the quantification of colonies, agar plates were prepared with medium produced as described beforehand (ASW) in two-fold concentration by using only 500 mL instead of 1 L of distilled water. 500 mL of distilled water were added mixed containing 5.0 g of peptone (Carl Roth, Karlsruhe, Germany), 1.0 g of yeast extract (Carl Roth, Karlsruhe, Germany) and 15.0 g of agar-agar (bacteriologically, C. Roth, Karlsruhe, Germany) and autoclaved. After autoclaving the agar and the 2x-ASW were combined, shaken and directly poured into sterile petri dishes under the clean bench.

2.1.4. Biological Material (Strains and Sediment Samples)

- ***Single Strains Rhodococcus sp. PC10 / PC20 & Halomonas sp. PC30***

Isolation of single strains from GoM sediments was performed by Xiaoxu Sun (School of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta, USA) during his master thesis. As a cooperation partner in the C-Image consortium he provided us with three of his isolates (PC10, PC20, PC30) for testing.

The isolation procedure was carried out with 2 g of sediment samples from two Gulf of Mexico sites PCB06 and DSH10. These were incubated with 0.5 % v/v MC252 oil in 20 mL of minimal artificial seawater medium (Widdel, 2010) at 4 °C. Incubations were transferred when the stationary phase was reached. In total, 26 *Rhodococcus* strains (PC1–2, PC 5–28) and two *Halomonas* strains (PC3, 4) were isolated from PCB06 sediment. Two strains of *Halomonas* (PC29, 30) were isolated from DSH10 sediment. Sanger sequencing results showed that the two *Halomonas* strains showed nearly a 100 % sequence identity with *Halomonas campaniensis*. All three *Rhodococcus* strains shared a 99 % sequence identity with *Rhodococcus erythropolis* and a cryo-stock of each strain was stored frozen at – 80 °C.

The culture of *Rhodococcus* PC20 strain (as well as PC10 / PC30) was kept on LB agar at 4 °C and transferred regularly (approx. every month).

- ***Sediment Samples***

Sediment was collected in August 2016 (WB0816) and August 2017 (WB0817) during the R/V WeatherBird II cruises. The five sediment coring sites were DWH01 at 1580 m depth, PCB06 at 1043 m depth, DSH08 at 1123 m depth, DSH10 at 1490 m depth, and SW01 at 1138 m depth (Tab. 2.4). Top sediment layers (0 - 1 cm) were used for this study. The sediments were collected and stored at 4 °C and frozen (–20 °C) and shipped to Hamburg University of Technology. The sediments were stored at 4 °C and frozen (–20 °C) before the start of the experiments.

Sample Name (cruise - site)	horizon (mm)	Core Lat. (N)	Core Long. (W)	Water depth (m)	Core Date	storage	Media
WB0816DWH01	0-10	28 43.462'	88 23.237'	1580	Aug 16	4°C	Sediment
WB0816DWH01	0-10	28 43.462'	88 23.237'	1580	Aug 16	frozen -20°C	Sediment
WB0816DSH08	0-10	29 18.44	86 40.495	1123	Aug 16	4°C	Sediment
WB0816DSH08	0-10	29 18.44	86 40.495	1123	Aug 16	frozen -20°C	Sediment
WB0816DSH10	0-10	28 58.764	87 53.480	1490	Aug 16	4°C	Sediment
WB0816DSH10	0-10	28 58.764	87 53.480	1490	Aug 16	frozen -20°C	Sediment
WB0816PCB06	0-10	29 53.935	86 17.68	1043	Aug 16	4°C	Sediment
WB0816PCB06	0-10	29 53.935	86 17.68	1043	Aug 16	frozen -20°C	Sediment
WB0816SW01	0-10	29 27.348	87 26.994	1138	Aug 16	4°C	Sediment
WB0816SW01	0-10	29 27.348	87 26.994	1138	Aug 16	frozen -20°C	Sediment
WB0817DWH01	0-10	28 43.462'	88 23.237'	1580	Aug 17	4°C	Sediment
WB0817DSH08	0-10	29 18.44	86 40.495	1123	Aug 17	4°C	Sediment
WB0817DSH10	0-10	28 58.764	87 53.480	1490	Aug 17	4°C	Sediment
WB0817PCB06	0-10	29 53.935	86 17.68	1043	Aug 17	4°C	Sediment
WB0817SW01	0-10	29 27.348	87 26.994	1138	Aug 17	4°C	Sediment

Tab. 2.4: Origin and depth of sediment samples used in experiments. Horizon is considered the depth below sediment surface where the sample was taken.

2.1.5. Incubation Schemes

As the sediment is a sample of a highly complex ecosystem, the procedures of incubating a complete microbial community as opposed to a single strain were quite different.

- ***Single Strain Experiments***

For the single strains (*Rhodococcus* PC20, *Cobetia* C6) a preculture was prepared each time before the start of the experiment. For this purpose, an inoculum loop of culture (from an agar plate) was dissolved (by vortexing and shaking) in 20 mL of medium in a crimp sealed bottle. For this, the substrate of the following experiment was added to the preculture. Growth of the preculture was monitored by taking 1 mL samples with a syringe and by measurement of the optical density (OD_{600}, SmartSpec Plus, BioRad, Germany) in comparison to a control (medium and substrate only, without inoculum). The preculture was repeated, if there was no change in the optical density or the final optical density was too low ($\Delta OD < 0.1$). Both precultures were kept at room temperature. For *Rhodococcus* PC20, experiments were started 48 – 72 hours after inoculation of the preculture. Precultures of *Cobetia* C6 were transferred after 96 – 168 hours.

To set up the incubation, the medium, substrate and preculture were mixed under sterile conditions under the clean bench. In autoclaved 100 mL glass vials (including a cleaned magnetic stirrer), first 20 mL of medium mixed with vitamin mix and trace element solution, then 1 mL of the preculture, and finally the substrate was added. The vials were covered with autoclaved aluminum foils and inserted into the pressure reactors. The aluminum foil was punctured with a sterile syringe several times right before closing the bronze lit. Before slowly building up pressure (approx. 3 MPa / min), the hose was flushed with nitrogen gas, at least once. After calibration of the oxygen sensors, the magnetic stirrers (Super-Nuova, Thermo Scientific, Germering, Germany) were set to 200 rpm (= t_0).

- ***Sediment Experiments***

Sediment experiments were treated differently in comparison to the single strain experiments. Incubations were started without a preculture, as this would have already been a selection step. Five of the sediment coring samples were used: DWH01 (1580 m bsl.), PCB06 (1043 m bsl.), DSH08 (1123 m bsl.), DSH10 (1490 m bsl.), and SW01 (1138 m bsl.) (Tab. 2.4). Over time, two batches of sediment samples were used (10-14: cruise Aug2016; 15-17: cruise Aug2017, Tab. 2.5) in different experiments. These sediments were weighted in equal amounts and a slurry was mixed with ASW (or FSW) medium. Under sterile conditions, this slurry was adjusted to contain 1 g wet weight of sediment per 20 mL incubation volume. Mixing was crucial to ensure optimal homogenization. After the slurry was transferred to the glass vials (20 mL to the 100 mL vials / 5 mL to the 10 mL vials) the substrate was added and the vials closed with autoclaved aluminum foil under sterile conditions. Substrates (Louisiana Light Sweet crude oil, dispersant EC9500A) were added directly to the aliquoted slurry with glass syringes (oil) and pipets (dispersant).

The procedure of insertion of the vials to the reactors and the subsequent pressurization were identical to the way described above for the single strain experiments. Tab. 2.5 shows an overview of all sediment experiments conducted. Experiments are referred to with their names or number in the following.

		date		medium		amount of sediment	pressure [MPa]			substrate		
#	name	start date	time [h]	name	[ml]	[g]	substrate		control	oil	Corexit	[µl]
10	**Oct16**	03.10.2016	1458	ASW	20	1.0	10	0.1	10	x	-	200
11	**Dec16**	03.12.2016	792	ASW	20	1.0	10	-	10	x	x	200
12	**Jan17**	10.01.2017	360	ASW	60	3.0	10	-	10	x	-	600
13	**May17A**	17.05.2017	168	ASW	20	1.0	10	0.1	0.1 / 10	x	-	200
14	**May17B**	26.05.2017	168	ASW	20	1.0	-	-	0.1 / 10	x	-	-
15	**Sept17A**	12.09.2017	168	ASW / FSW	20	1.0	10	0.1	0.1 / 10	x	-	200
16	**Sept17B**	26.09.2017	168	FSW	5	5 ml slurry (1g /20 ml)	10	0.1	10	x	x	50 / 2
17	**Feb18**	21.02.2018	168	FSW	5	5 ml slurry (1g /20 ml)	10	0.1	0.1 / 10	x	x	50 / 2

Tab. 2.5: Overview of the experiments conducted with subsequent NGS analysis. ASW = Artificial seawater; FSW = filtered seawater. Fourth column refers to the pressures under which experiments either with addition of substrate (substrate) or without substrate (control) were conducted.

2.2. Analysis of Experimental Runs

In the following the data acquisition and downstream analysis is described for all experiments conducted.

2.2.1. Direct Measurements

Direct measurement are considered those started directly after the incubation without any further experimental steps (e.g. extraction steps).

- ***pH***

After most experiments the pH of the medium was measured and compared to the initial pH and pH of the pure medium. Measurements were conducted in a 1.5 mL reaction tubes (Eppendorf) with the SCHOTT CG 822 (Schott AG, Mainz, Germany) pH meter.

- ***Optical Density***

To determine growth during the incubations the optical density of the incubation medium was measured. The optical density at a wavelength 600 nm increases linearly with cell density. To not overestimate the cell density the Spectrophotometer (SmartSpec Plus, BioRad, München, Germany) was calibrated with the pure medium. No attempt was made to link the OD_{600} value to cell numbers, as values are highly biased by crude oil droplets in the incubation medium.
In 1.5 mL cuvettes, initially the medium was introduced to the measuring chamber and reset to zero. Subsequently the samples were measured, after inverting the sample several times.
Crude oil is forming an emulsion in (sea) water, and therefore interferes irregularly with the measurements. Hence, these measurement results with crude oil as substrate have to be evaluated critically.

- ***Cell Numbers***

To measure cell concentrations more precisely colony forming units (CFU) were measured. The principle of the method is to take a certain amount of incubation solution (10 µL) and spread plate it in replicates (n = 3 – 4) on a solid medium (LB / MA) in a distinct spot. This has to be repeated in different dilution steps (n = 4), to ensure colony counts between one and 20 colonies in a 10 µL spot. This investigated dilution window had to be adjusted in the course of the incubation, due to growth.

For *Rhodococcus* sp. PC20 LB agar plates were used, whereas MA plates were used to count *Cobetia* sp. C6 / C8 and the sediment colonies.

2.2.2. Extraction and DNA Concentration Measurement

Further analysis on sediment experiments and the incubated community of microorganisms depended on extracting the 16S rRNA from the samples and verifying the successful extraction step before sending the samples for sequencing.

Total DNA was extracted from sediment slurries with MoBio PowerSoil DNA Isolation Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. Adaptations to the wet soil sample were made according to the Hints and Troubleshooting Guide (MoBio Power Soil DNA Isolation Kit, version 02232016, QIAGEN, Hilden, Germany) by centrifuging the sediment two times 2 mL and discarding the supernatant. Extracted DNA was stored at - 20 °C until further use.

Before sending the DNA for sequencing, the concentration of genetic material per sample had to be determined. Fluorescence concentration measurement was performed with a Promega Quantus Flourometer (Mannheim, Germany) and the QuantiFlour dsDNA Sample Kit (Promega, Mannheim, Germany). A negative control of sterile water instead of DNA extract solution was included in the measurement series.

2.2.3. Amplicon Sequencing and Data-Preprocessing

Paired end amplicon sequencing of V3 and V4 variable regions of the 16S rRNA gene were performed on 300 bp paired-end read Illumina MiSeq platform using 341F (CCTACGGGNGGCWGCAG, Muyzer et al., 1993) and 785R (GACTACHVGGGTATCTAAKCC, Klindworth et al., 2013) primers at LGC Genomics (Berlin, Germany).

The following data pretreatment was conducted by LGC (Berlin, Germany). All libraries for each sequencing lane were demultiplexed using the Illumina bcl2fastq 2.17.1.14 software (bcl2fastq, v2.19.1). All reads were sorted by amplicon inline barcodes and the barcode sequence was clipped afterwards. Subsequently a clipping of sequencing adapter remnants from all reads was conducted, whereby reads with a final length of < 100 bases were discarded. Finally, forward and reverse primers were detected and clipped off the reads.

2.2.4. Analysis of Sequence Data With QIIME2

QIIME2 is an open source bioinformatical tool (Quantitative Insights Into Microbial Ecology, Caporaso et al., 2010), which allows analysis of high-throughput community sequencing data on basis of marker gene (16S / 18S rRNA) amplicon sequencing. In the following the version and steps taken to analyze the sequenced data are introduced. QIIME2 command inputs are italicized.

- ***Version***

For this analysis the QIIME2 version 2018.8 was run on the Oracle VM VirtualBox (Version 5.2.18 r124319, Qt5.6.2) on an Ubuntu64 operating system.

- ***QIIME2 Pipeline Steps***

The reverse and forward paired-end reads were assembled, demultiplexed, chimera filtered and quality filtered using DADA2 (Callahan et al., 2016) with a quality score ≥ 27 (*p-trim-left* 0 / *p trunc-len* 231). Sequences were clustered into sequence variants and representative sequences (similar to OTU, 97% sequence identity) were given as an output.

After creating a rooted tree for phylogenetic analysis from the representative sequences (QIIME2 command succession: *qiime alignment mafft, qiime alignment mask, qiime phylogeny fasttree, qiime phylogeny midpoint-root*), the core-metrics-phylogentic method (with a sampling depth of 10^5) was run to obtain several alpha diversity indices (Shannon's diversity, Observed OTUs, Faith's Phylogenetic Diversity, Pielou's Evenness) and beta diversity distance matrices (Jaccard, Bray-Curtis, unweighted UniFrac, weighted UniFrac).

To assign the taxonomy to the sequences, first a classifier was trained (naive-bayes; 231 bases) with the primers 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAAKCC) on the latest Greengenes database release (latest release 13_8, McDonald et al., 2012) with the

16S rRNA 99 % sequence identity. This classifier was applied to the representative sequences (*qiime feature-classifier classify-sklearn*) to obtain the taxonomical assignment.

▪ *Statistical Methods*

Several statistical analyses were performed in this study. To test whether different samples (here: from different days, Tab. 3.1) originate from the same distribution the non-parametric Kruskal Wallis Test was used (Kruskal and Wallis, 1952), which operates on ranks and enables more than just pairwise comparisons. Every non-parametric test on just two groups (pairwise) was performed using the Wilcoxon Rank Sum Test (Mann and Whitney, 1947). Those test were used (Kruskal Wallis Test, Wilcoxon Rank Sum Test) as the data sets were not normally distributed.

The alpha diversity of a sample is defined as the distribution of taxa within a sample (habitat), and can be calculated by different indices. The Shannon index (Shannon, 1948) is a quantitative measurement for the community richness, which incorporates the relative abundance of each taxon and the total number of species present. The higher the Shannon index the higher the diversity. Counting the observed OTUs (operational taxonomic unit) gives a qualitative measurement of the diversity as the amount of OTUs present in the samples. Faith's index (Faith, 1992) evaluates the phylogenetic diversity, by incorporating the position of each taxon on the phylogenetic tree. The resulting value give the sum of all branch lengths connecting the species in the sample's community, which means a higher value represents a higher phylogenetic diversity. Vice versa, the lower the Faith's index the closer phylogenetic relation of taxa in the sample. The Pielou's Evenness index (Pielou, 1996) measures the samples community eveness using the Shannon index divided by the maximum possible diversity value. This gives a value between 0 (completely uneven distributed community) and 1 (uniform distributed community).

Beta diversity describes the diversity comparing different samples (habitats). To evaluate beta diversities Principal Coordinates Analysis (PCoA, Gower, 1966) plots were created using the Bray-Curtis dissimilarity matrix with the EMPEROR QIIME2 online plugin (2018.11.0) to represent dissimilarity between samples in a low-dimensional space. Bray-Curtis dissimilarities (Bray and Curtis, 1957) are calculated from pairwise comparison of two samples, where the number of the species in common between the two samples is divided by the sum of all species. This value is subtracted from 1, which means dissimilarities range between 0 (same composition of species) and 1 (no species in common). PCoA plots are used to visualize complex ecological data by displaying relations between samples. In a two dimensional plot, similar samples group together, while highly dissimilar samples are separated. To test differences among treatment groups for significance a Permutational Analysis of Variances (PERMANOVA, Anderson, 2001) was performed using as well the Bray-Curtis dissimilarities (999 permutations). Differentially abundant taxa were identified across samples with the Analysis of Composition of Microbiomes (ANCOM, Mandal et al. 2015). Differences among the eight treatment groups (by pressure/substrate: control 0.1MPa, control 10MPa, oil 0.1MPa, oil 10MPa, disp 0.1MPa, disp 10MPa, d.oil 0.1MPa, d.oil 10MPa) were investigated with an added pseudocount (*qiime composition add-pseudocount*, *qiime composition ancom*). The features were collapsed at the taxonomic level 6 (genus, *qiime taxa collapse*).

To evaluate the impact of the applied parameters on the community composition, a linear model was created on the balances instead of proportions (relative abundances) with gneiss balances following the instructions given in the QIIME2 tutorial (https://docs.qiime2.org/2018.2/tutorials/gneiss/, accessed March 2019). Using balances account for the problem of compositionality, which means an absolute change in abundant reads of a single taxon changes not only the proportion of the changing taxon, but to a smaller degree those of all other taxa. In brief, for gneiss correlation clustering a phylogenetic tree was created. This composition artifact was transformed with the *gneiss ilr-hierarchical* function to calculate gneiss balances at each internal node with the isometric log ratio (ILR) transformation. The ordinary least squares (OLS) model used these gneiss balances to calculate the parameters' influences on the variances of the communities' structure. The formula for the OLS model (*qiime gneiss ols-regression*) was constructed with the parameters (factors) "oil", "pressure" and "dispersant" as binary data.

2.3. Isolation Procedure to Obtain *Cobetia* Strains

In this chapter the isolation procedure is described, which lead to the isolation of eight *Cobetia* strains.

2.3.1. Experimental Conditions

As a start of isolation, the incubation with 200 µL of crude oil in 20 mL of ASW of the May17A (Exp. 13) experiment was used. Before pressurizing with nitrogen gas to 10 MPa, 1 MPa of methane was added to the reactor. After incubation for 7 days at 4 °C, from one replicate of the incubation with crude oil (and methane) 100 µL were spread plated on marine agar (MA). The plate was incubated at 4 °C for one week.

After colonies formed on the MA plate, eight of them were picked, transferred to another plate and incubated at 4 °C for another week. The 16S rRNA of the eight isolates (C1 – C8) was amplified with colony PCR. The reaction mixture consisted of 10 µL of 10-fold concentrated PCR buffer (QIAGEN, Hilden, Germany), 6 µL of $MgCl_2$ (QIAGEN, Hilden, Germany), 2 µL of 10 mM dNTP mix (peqGold, PEQLAB Biotechnologie GmbH, Erlangen, Germany) and 0.1 µL of each primer solution (100 pmol/µL; 341F-GC clamp (Schäfer and Muyzer 2001), 907R (Lane, 1991)), 1 µL of Taq polymerase (5 U/µL, peqGold, PEQLAB Biotechnologie GmbH, Erlangen, Germany), 2 µL of template and filled up to 100 µL per reaction. Testing 5 µL of the resulting mixture on 1 % agarose gel (LE Agarose, Biozym, Hessisch Oldendorf, Germany) with 6x loading dye (DCode 2x loading dye, Bio-Rad, München, Germany), run for 15 min at 100 V. The presence of PCR products was visualized using 10 x SYBR-Green (Lonza, Rockland, ME, USA) and a UV table (PEQLAB ECX-20.M, PEQLAB Biotechnologie GmbH, Erlangen, Germany) and excluded false positive signals by including a negative control in the run. The remaining PCR reaction mixture was purified with a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and the product tested again on a 1 % agarose gel for products. Afterwards the samples were send for Sanger Sequencing (GATC Eurofins Genomics, Ebersberg, Germany). For the PCR a thermocyler (Primus 25 advanced®, PEQLAB, Biotechnologie GmbH, Erlangen, Germany) was used with the program described in Tab. 2.6.

Step		T (°C)	Time		Cycles
1	denaturing	94	5	min	1
2	denaturing	94	30	sec	30
	annealing	55	1	min	
	elongation	72	1,5	min	
3	elongation	72	10	min	1
4	hold	8			

Tab. 2.6: PCR program for amplification of 16S rRNA (colony PCR / after purification).

2.3.2. Culture Maintenance

Cobetia cultures were kept on MA plates at 4 °C in the fridge and were transferred after visible growth (approx. every 40 – 60 days) with the 13-streak method for isolating single colonies on an agar plate.

3. Results

3.1. *Rhodococcus sp. PC20* – the Effect of Pressure and Dispersant on the Growth of a Single Strain Isolated From the Gulf of Mexico (GoM)

Parts of this chapter are published in Hackbusch, S., Noirungsee, N., Sun, X., Viamonte, J., Kostka, J., Bubenheim, P., Müller, R., Liese, A. (2019). Crude oil degradation performance of a deep sea *Rhodococcus* PC20 strain under the influence of elevated hydrostatic pressure and dispersant. Marine Pollution Bulletin.

3.1.1. Isolation Procedure and Characterization of GoM Isolates

Our cooperation partner, Xiaoxu Sun (School of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta, USA), shipped to the Institute of Technical Biocatalysis three of the strains (PC10 / PC20 / PC30) he had isolated from the northern GoM deep sea sediment with crude oil as substrate (0.5 % v/v) at atmospheric pressure (Sun, 2014). As a first step, preliminary experiments were conducted to check for the strain's ability to degrade crude oil and to determine the most promising of these strains, regarding growth rates. Further, the optimal growth medium as well as the ideal concentration of crude oil for the chosen strain were investigated.

- ***Cultivation Conditions of Rhodococcus sp. Isolates***

Rhodococcus sp. PC10 / PC20 /PC30 were isolated from deep sea sediment near the DWH well and have been among the fastest growing strains under the conditions applied during the isolation procedure from GoM sediment (Sun, 2014). The following selective parameters, like atmospheric pressure, room temperature, crude oil as substrate, already give insight into the growth preferences of the strain and the dominance in the cultures highlights its hydrocarbon degradation potential. However, those assumptions had to be confirmed and the strain's cultivation conditions were evaluated.

With a crude oil concentration of 0.1 % v/v, PC10 and PC20 were able to grow exponentially within the first 68 h (μ_{PC20} = 0.04 h^{-1}, μ_{PC20} 0.05 h^{-1}). While PC30 was unable to increase substantially in cell numbers during this time period (μ_{PC30} = 0.01 h^{-1}) (Fig. 3.1).

Based on these results the decision was made to pick *Rhodococcus* sp. PC20 for further experiments.

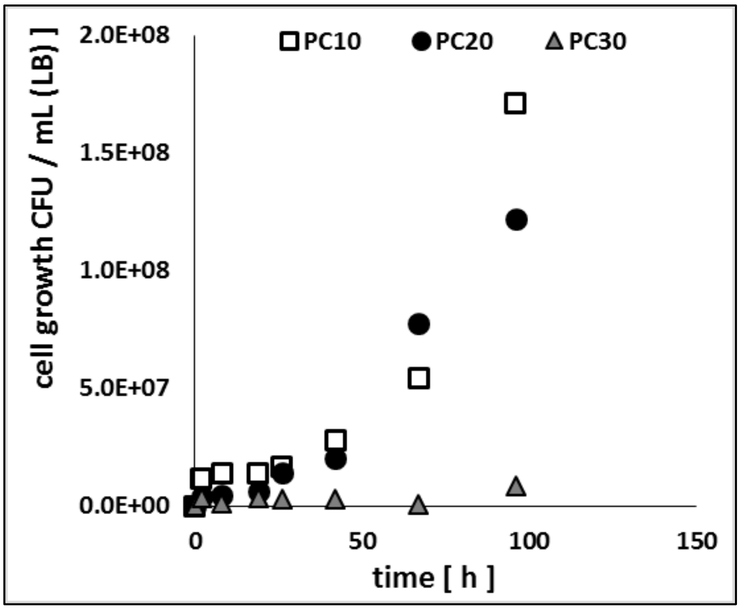


Fig. 3.1: Cell growth (CFU mL^{-1}) of Rhodococcus *sp. PC10 / PC20 / PC30 in 50 mL MM2 medium for 96 h with crude oil (0.1 % = 50 µL) at 0.1 MPa, RT, 200 rpm.*

- ***Nutrient Requirements of Rhodoccocus sp. PC20***

Biodegradation of crude oil depends on the abiotic factors like hydrocarbon transport, oxygen and nutrient availability (Head, 2006). Nitrogen and phosphorus containing salts and organic compounds are important nutrients, which are depleted in crude oil components. Especially offshore pelagic habitats are limited in supply of these nutrients (Kleindienst et al., 2015). Therefore, growth in Minimal Mineral medium (MM2) was compared to the artificial seawater medium (ASW, Widdel, 2010) and sterile filtered seawater (FSW, Fig. 3.2). MM2 contains higher concentrations of nitrogen and phosphorus containing salts and organic compounds (15.1 / 28.1 mM, respectively) in relation to ASW (20.0 / 2.0 µM, respectively).

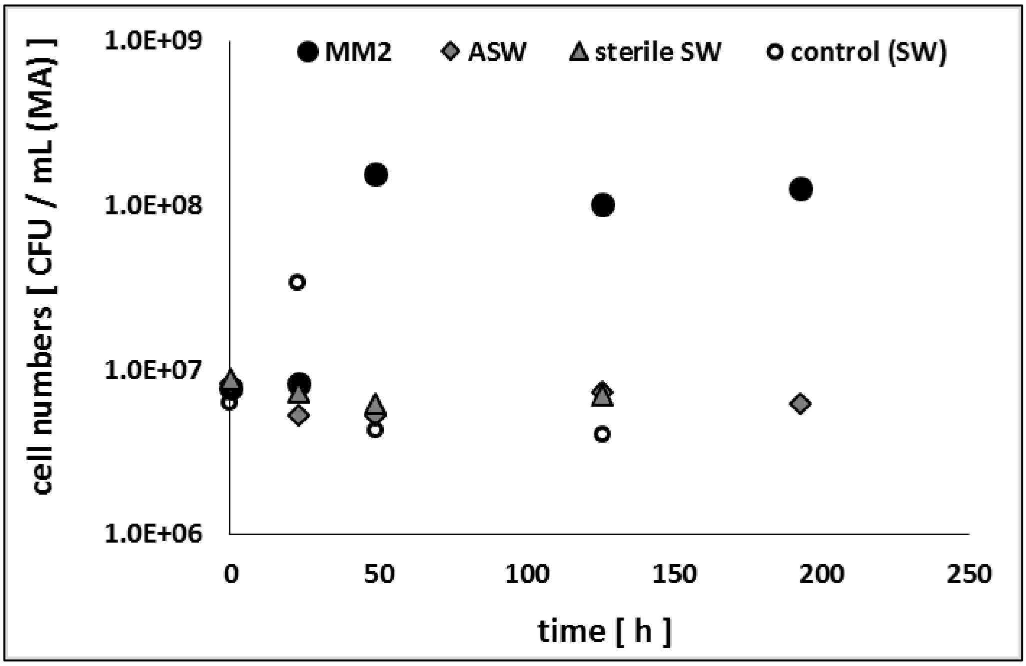


Fig. 3.2: Cell numbers of Rhodococcus *PC20 incubated with 40 mL of different media (MM2, ASW, sterile seawater (sterile SW/FSW)) and 40 µL of crude oil (0.1% v/v), at 0.1 MPa, RT for 193 h. The growth control (control (SW)) was an incubation in sterile filtered seawater (FSW) without any crude oil added.*

The medium, recommended by the collaboration partner Xiaoxu Sun (ASW), was not able to support growth of *Rhodococcus* sp. PC20. MM2 was the only medium supporting positive growth rates ($\mu_{MM2} = 0.02\ h^{-1}$), while incubations in ASW and FSW did not show any growth ($\mu_{ASW/FSW} < 0.00\ h^{-1}$). According to these findings, MM2 was used in all subsequent experiments with *Rhodococcus* sp. PC20.

The isolation conditions were set to be as close as possible to the habitat where the samples were retrieved. In contrast to this, the aim of high pressure experiments was a high throughput of experiments. These different rationales led to the decision to change from the ASW medium to MM2 for high pressure experiments, as the strain demonstrated an increased growth with the MM2 medium.

▪ *Effect of Crude Oil Concentration on Rhodococcus sp. PC20*

The strain was isolated with 0.5 % v/v of crude oil to medium ratio. Whether an increase in crude oil concentration would lead to higher growth rates or would be toxic to the strain had to be investigated. The relative amounts of crude oil to media ratio (0.1 / 0.5 / 1 % v/v) were investigated to find the growth limits for *Rhodococcus* sp. PC20. The minimum concentration of crude oil needed for the detection of significant growth and a possible upper limit of oil, where crude oil components would become toxic to *Rhodococcus* sp. PC20, were checked (Fig. 3.3).

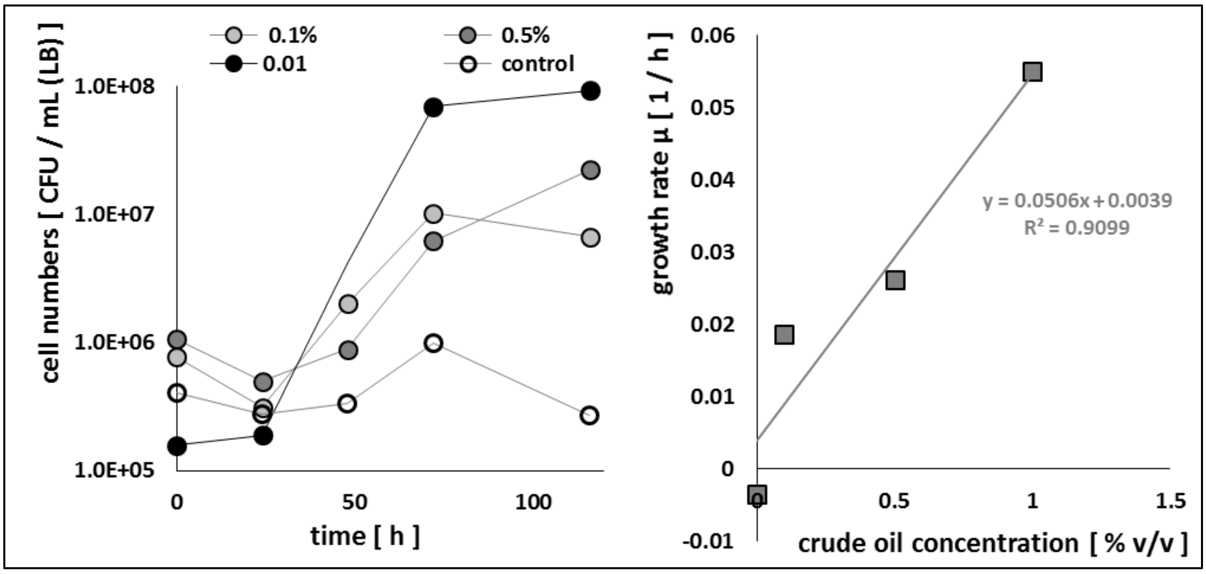


Fig. 3.3: Left: Cell concentration [CFU mL^{-1}] of Rhodococcus *sp. PC20 incubated with MM2 medium and different volumetric concentrations of crude oil (0.1 % / 0.5 % / 1 %) for 116 h at 0.1 MPa, RT, 200 rpm. Right: Correlation of growth rates [h^{-1}] after 116 h of incubation with different substrate amounts [% v/v].*

Rhodococcus sp. PC20 was able to grow with any amount of crude oil added to the incubation (range μ_{116h}: 0.019 – 0.055 h^{-1}). In addition, the results revealed a positive linear correlation of substrate amount and growth rates (0 - 116 h (stationary phase)) in the tested range of substrate concentration (0.1 – 1 % v/v). Therefore, 1 % v/v was chosen as the default amount of crude oil in the following incubations.

The amount of crude oil added as substrate to the incubation was positively correlated with the growth of *Rhodococcus* sp. PC20, in the range between 0.1 and 1 % v/v of crude oil to volume of medium (Fig. 3.3 right). Within the chosen operational window, these findings show that *Rhodococcus* PC20's growth is not limited by the nutrients available and is linearly correlated with the substrate amount.

3.1.2. Substrate Affinities of *Rhodococcus* sp. PC20

It has been shown, that *Rhodococcus* sp. PC20 could thrive on crude oil, without any information on the compound classes (saturated aliphatic or aromatic compounds). Individual carbon sources (LB, *n*-hexane, *n*-heptane, *n*-decane, *n*-hexadecane, benzene, naphthalene) were investigated regarding the ability to support growth of *Rhodococcus* sp. PC20. A highly specialized usage of carbon sources could already be ruled out at this point, as crude oil is a very complex mixture of hydrocarbons. Subsequently, *Rhodococcus* PC20 was tested on its ability to grow on another highly complex organic medium (Lysogeny Broth, LB, Fig. 3.4). Growth on LB was equally assumed, as the culture was kept on and transferred to LB agar plates.

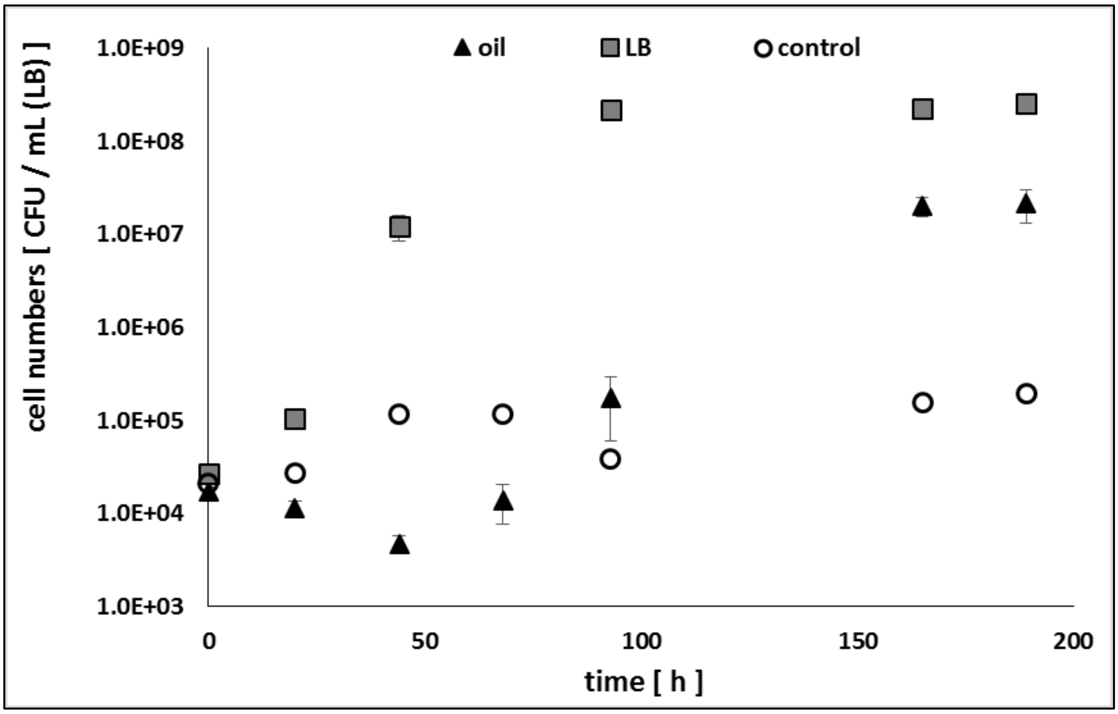


Fig. 3.4: Cell numbers [CFU mL^{-1}] of Rhodococcus *sp. PC20 incubated with MM2 medium and different substrates: LB and crude oil (1 % v/v) for 189 h at 0.1 MPa, RT, 200 rpm. As control an incubation without substrate added was used.*

Growth was observed for both substrates amended (LB / crude oil). Incubations with crude oil showed a longer lag phase (~ 68 h) compared to LB (~ 20 h) and while higher maximum growth occurred with LB (LB: 2.5*10^{8} CFU mL^{-1}; crude oil: 2.1*10^{7} CFU mL^{-1}). Therefore, LB can be seen as the preferred substrate.

Due to difficulties to accurately compare LB and crude oil concentrations, the substrates were standardized in the following experiments to the carbon concentration of each substrate (to 16 mmol C L^{-1}).

These findings suggest a generalist usage of organic carbon by *Rhodococcus* sp. PC20, as LB is a complex mixture of organic compounds. Further, crude oil seems not to be the ideal (complex) substrate for *Rhodococcus* sp. PC20, as longer lag time, lower growth rate and lower maximum cell concentrations were observed in incubations on crude oil compared to LB.

Rhodococcus sp. PC20 was able to grow on crude oil, which consists of a wide variety of chemical compounds. A more detailed growth analysis of the strain on the compounds crude oil compounds (*n*-hexane, *n*-heptane, *n*-decane, *n*-hexadecane, benzene, naphthalene) was performed in addition to crude oil (Fig. 3.5) to detect preferences.

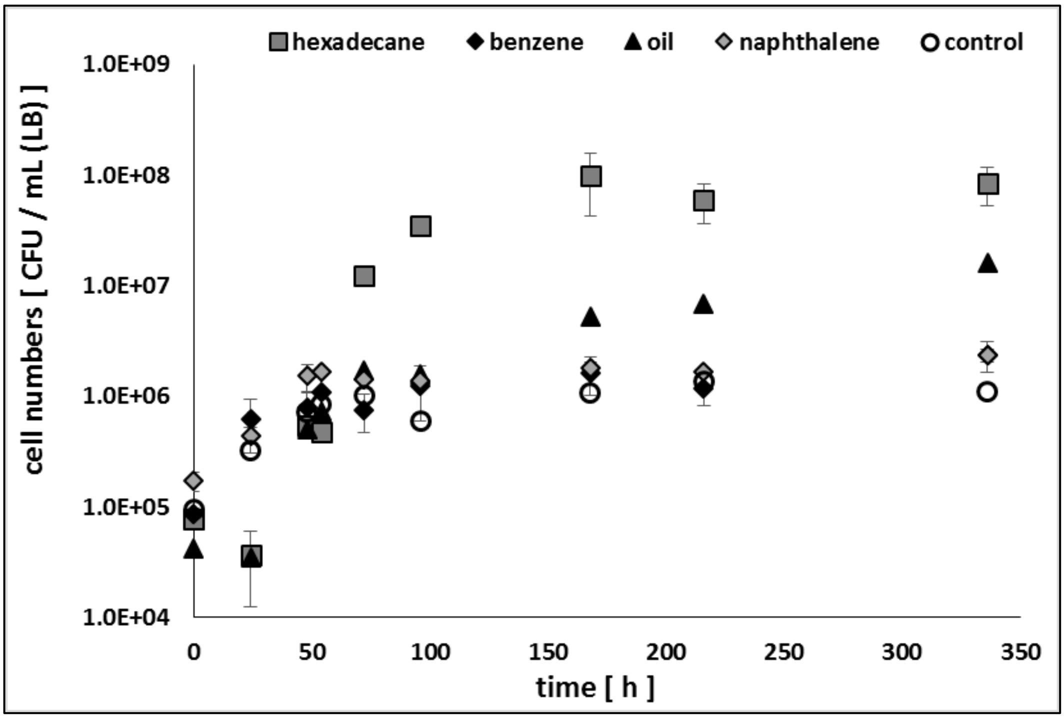


Fig. 3.5: Cell numbers [CFU mL^{-1}] of Rhodococcus *sp. PC20 incubated with 20 mL MM2 medium and different substrates (n-hexadecane [1.0 mmol/L], benzene [2.7 mmol/L], crude oil (5.2 µL = 0.025% v/v), naphthalene [1.6 mmol/L]) for 600 h, 0.1 MPa, RT and 200 rpm. Substrates normalized to 16 mmol carbon per liter. Experiment J1 (Tab.2.2), n = 2.*

Only hexadecane led to a higher growth rate (μ_{168h}: 0.043 h^{-1}) compared to crude oil (μ_{168h}: 0.029 h^{-1}). None of the aromatic compounds (benzene / naphthalene; μ_{168h}: 0.018 / 0.014 h^{-1}) supported growth significantly, in comparison to the growth control (without substrate; μ_{168h}: 0.014 h^{-1}).

This insignificant growth on mono- and polyaromatic components (benzene and naphthalene) points out the inability of *Rhodococcus* sp. PC20 to substantially metabolize aromatic carbon sources.

Only the saturated *n*-alkanes did support growth of *Rhodococcus* sp. PC20. Therefore, the effect of chain length of *n*-alkanes (*n*-hexadecane, *n*-hexane, *n*-heptane, *n*-decane) as substrate on the growth was investigated (Fig. 3.6).

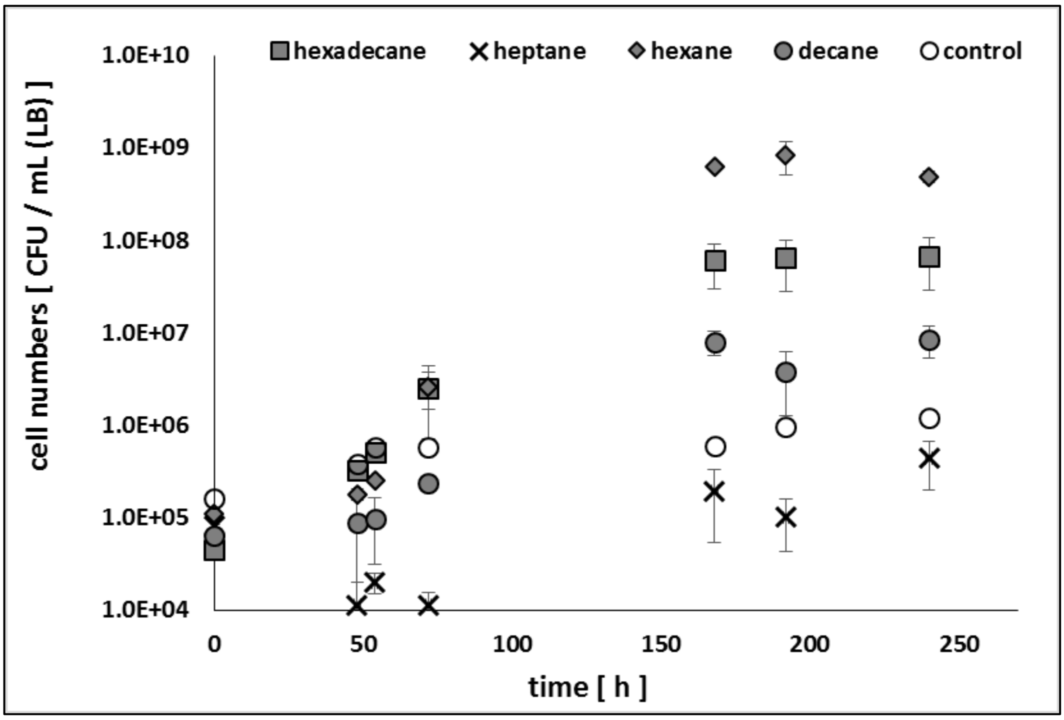


Fig. 3.6: Cell numbers [CFU mL^{-1}] of Rhodococcus *sp. PC20 incubated with 20 mL MM2 medium and different substrates (*n*-hexadecane [10.0 mmol/L],* n*-hexane [26.7 mmol/L],* n*-heptane [22.9 mmol/L],* n*-decane [16.0 mmol/L]) for 240 h, 0.1 MPa, RT and 200 rpm. Substrates normalized to 160 mmol carbon per liter. Exp. J2 (Tab.2.2).*

All substrates with even chain length (*n*-hexadecane, *n*-hexane, *n*-decane) were able to support growth of *Rhodococcus* sp. PC20. Hereby, highest growth rates were yielded with *n*-hexane (μ_{168h}: 0.051 h^{-1}), while *n*-heptane did not support growth at all (μ_{168h}: 0.005 h^{-1}), with lower growth rates than those of the biotic control (μ_{168h}: 0.008 h^{-1}). Incubations with *n*-decane and *n*-hexadecane resulted in significant growth rates (μ_{168h}: 0.029 / 0.043 h^{-1}, respectively). Nonetheless, no correlation between growth values (growth rates as well as maximum CFU ml^{-1}) and chain length can be deduced with too few substrates tested. A more detailed study needs to be carried out.

There is no obvious pattern detectable in the preferences to grow on certain *n*-alkanes (*n*-C6 > *n*-C16 > *n*-C10 > control > *n*-C7). Heptane was the only compound not supporting growth in *Rhodococcus* sp. PC20. However, as *n*-heptane is the only aliphatic compounds tested with an odd C-number, there is no general trend in respect to chain length deducable. DeCarvalho et al. (2009) found significant differences in the utilization of odd and even chain length *n*-alkanes, when investigating a correlation between chain length degradation and the fatty acid production in cell membranes by a *Rhodococcus erythropolis* strain.

3.1.3. *Rhodococcus sp.* PC20 at Ambient and Elevated Pressure

Rhodococcus sp. PC20 was isolated from deep sea sediments under atmospheric pressure conditions. The varying pressure conditions of the original habitat (~11 MPa) and during the isolation procedure (0.1 MPa) of *Rhodococcus* sp. PC20 implies the ability to not only endure both pressures, but being able become dominant during the isolation procedure. Due to this observation alone, the strain cannot be assumed to be a piezophilic, but a piezotolerant organism.

In total 20 experiments were conducted to compare the growth of *Rhodococcus* sp. PC20 at ambient (0.1 MPa) and elevated pressure (15 MPa), each with the same pre-culture (Fig. 3.7). The highest technically feasible pressure was chosen (15 MPa) to maximize possible pressure effect on the strains behavior. Incubations at atmospheric pressure showed that the growth of PC20 was not depending on elevated pressure (piezophily).

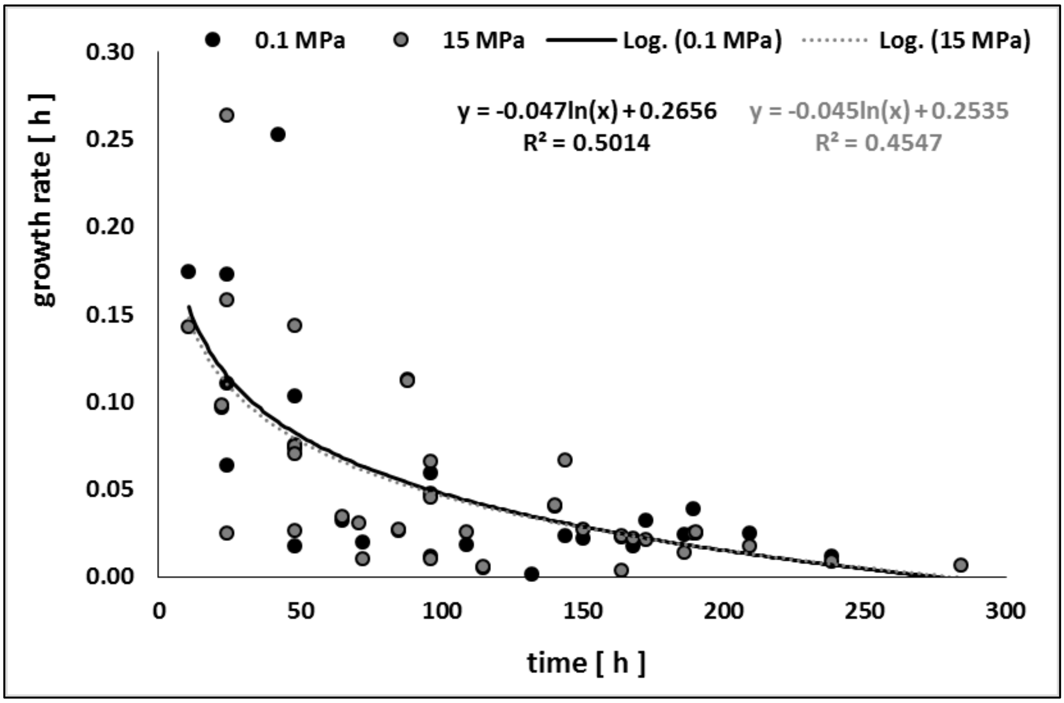


Fig. 3.7: Growth rates of all experiments conducted (n = 20), with crude oil (1 % v/v), for different incubation times up to 284 h, at both pressures (0.1 and 15 MPa). Logarithmical regression (Log.) indicated in black (0.1 MPa) and dotted (15 MPa) line.

Changes in CFU during incubations at both pressures can be described with a logarithmical function over time. This is showing the typical bacterial growth phases, including lag, exponential, and static phase. The net growth in all atmospheric pressure experiments ranged from $2.2*10^6$ to $5.6*10^9$ CFU mL^{-1} while in all elevated pressure experiments from $4.9*10^6$ to $3.0*10^{10}$ CFU mL^{-1}. Median growth did not differ significantly among both pressures with $1.7*10^8$ CFU mL^{-1} (0.1 MPa) and $2.1*10^8$ CFU mL^{-1} (15 MPa). Even though preculture conditions were kept constant, starting concentrations varied between $1.1*10^5$ and $1.8*10^8$ CFU mL^{-1}. Growth rates calculated from all incubation times were compared to the starting concentrations and revealed no significant difference between the incubations at both pressures (Wilcoxon rank sum test, alpha = 0.05, p = 0.049).

Some of these growth experiments at two pressures the oxygen concentration gaseous phase of the reactor were monitored by online measurement of oxygen. Exemplarily, four averaged experiments investigating different pressures on the growth of *Rhodococcus* sp. PC20 on crude oil are shown in Fig. 3.8.

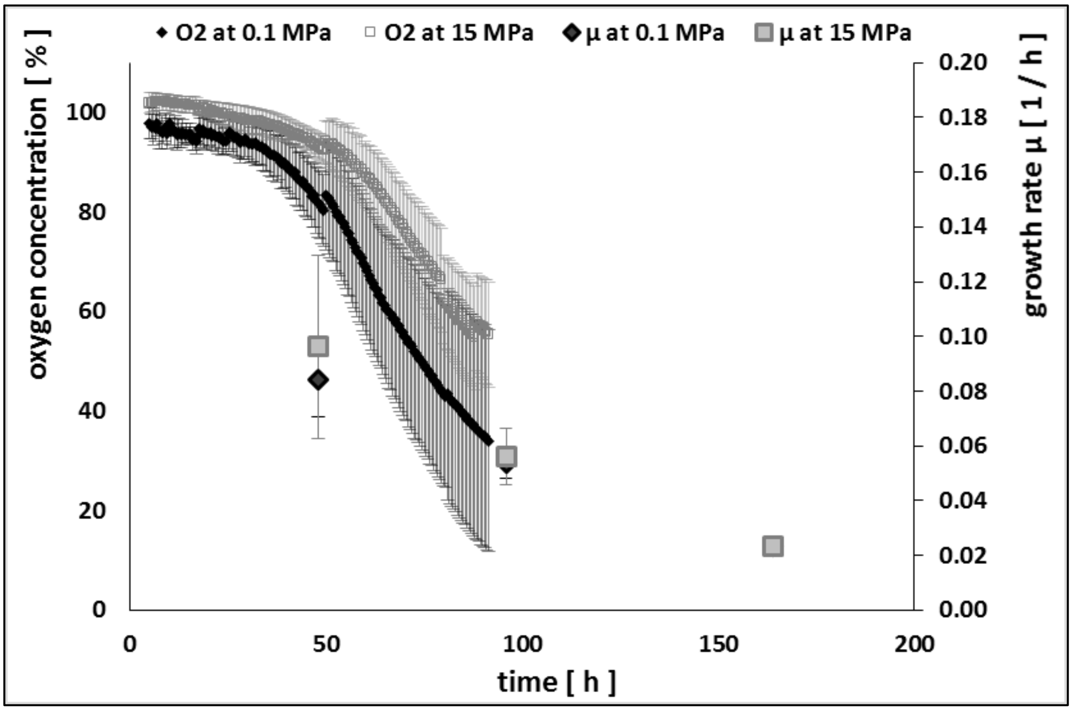


Fig. 3.8: Oxygen concentration [%] (two point calibration) in the reactors headspace and growth rates [h^{-1}] of Rhodococcus *sp. PC20 with 20 mL MM2 medium, crude oil (1 % v/v) at RT, 200 rpm for 164 h. n = 4.*

Rhodococcus sp. PC20 consumed oxygen quicker at atmospheric pressure than at elevated pressure, but with high uncertainties (SD) detected. Different growth rates measured for distinct time points of these incubations showed decreasing growth rates over time, which did not differ significantly. Oxygen consumption rates indicate a complete consumption of oxygen in the course of the incubation.

Growth rates of *Rhodococcus* sp. PC20 at atmospheric and elevated pressure (Fig. 3.7) did not resulted in a significant difference, regardless of the incubation time. This means *Rhodococcus* sp. PC20 is a piezotolerant bacterial strain, which is not influenced by pressures equivalent to the DWH well depth. It would be interesting to know, whether higher pressures, common in the deep sea (30 – 40 MPa), would affect the growth of this strain on crude oil components.

Measurements of oxygen consumption by *Rhodococcus* sp. PC20 generally confirmed, qualitatively, a not significant difference between performances at both pressures. However, due to very high standard deviations, the applicability of this measurement is questionable. Factors like different initial cell numbers and the sensitivity of the oxygen sensor at elevated pressure limit the comparative quantitative measurement.

3.1.4. Effect of Dispersed Crude Oil on the Growth of *Rhodococcus* sp. PC20

During the period of the uncontrolled hydrocarbon flow from the DWH well, about three million liter of the dispersant Corexit EC9500A were applied directly to the jet forming above the well in the deep sea to increase bioavailability of the crude oil by an increased oil surface area. There is still an ongoing debate about the efficiency of chemically dispersed crude oil and its effect on deep sea organisms (Kleindienst et al., 2015, Prince et al., 2016). Overholt et al. (2016) reported species-specific reactions of two hydrocarbon degrading bacteria to the addition of Corexit EC9500A.

Experiments with different ratios of dispersant to oil (DOR) were conducted to elucidate, whether the dispersant enhances biodegradation of crude oil by *Rhodococcus* sp. PC20, in this chapter only at atmospheric pressure. The dispersant was added directly to the incubation.

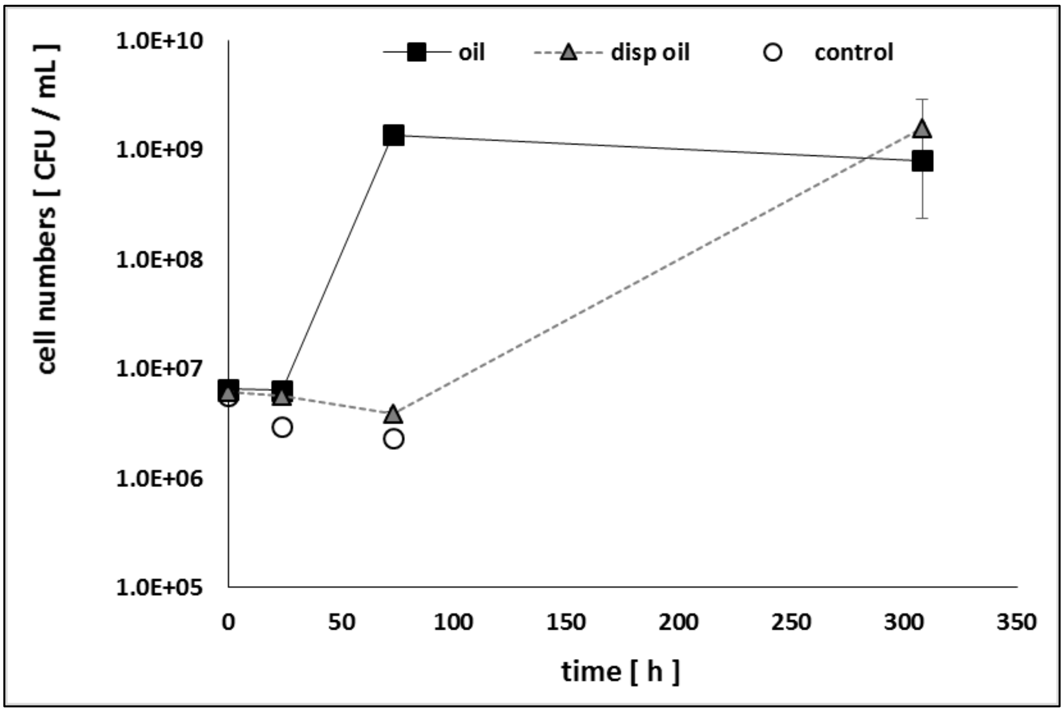


Fig. 3.9: Cell numbers [CFU mL^{-1}] of Rhodococcus *sp. PC20 in 30 mL MM2 with 300 µL crude oil and 0.3 µL dispersant (DOR = 1:1000) for 308 h at atmospheric pressure (0.1 MPa), RT, 200 rpm. n = 2. Lines connecting the measured points are for visual aid.*

Initially, dispersant was added to the incubation of *Rhodococcus* sp. PC20 with crude oil in a DOR of 1:1000. Results showed no effect on the overall growth of PC20, but a delay in entering the exponential phase with the addition of dispersant. The presence of dispersant did not affect the maximum cell numbers of *Rhodococcus* sp. PC20 (Fig. 3.9).

Multiple dispersant to oil ratios (DORs) were tested in order to check for an upper limit of dispersant regarding growth of *Rhodococcus* sp. PC20 (Fig. 3.10). A decrease in CFU per mL with a DOR of 1:100 and higher (1:20) was detected. A DOR of 1:400 (0.5 µL to 200 µL crude oil) showed neither growth nor decrease of CFU per mL. Starting concentrations of this particular experiment were comparably high (average: 5.4*10^7 CFU mL^{-1}).

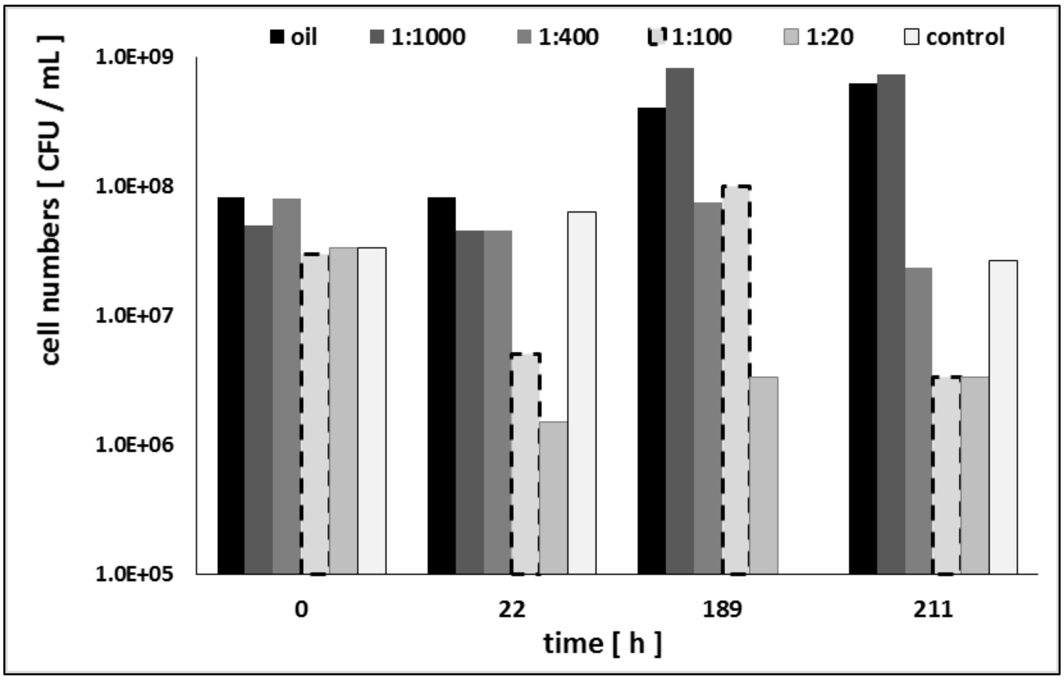


Fig. 3.10: Cell concentrations [CFU mL^{-1}] of Rhodococcus *sp. PC20 in 20 mL MM2 with 200 µL crude oil and varying concentrations of dispersant (0, 0.2, 0.5, 2 and 10 µL) at 0.1 MPa, RT, 200 rpm. n = 1.*

As starting concentrations were varying among experiments, each experiment was analyzed separately. In a repetition of the experiment described before (Fig. 3.11), it could be confirmed that a DOR of 1:100 lead to a relative decrease in CFU and the delay in growth with a DOR of 1:1000, in comparison to the incubation with crude oil only.

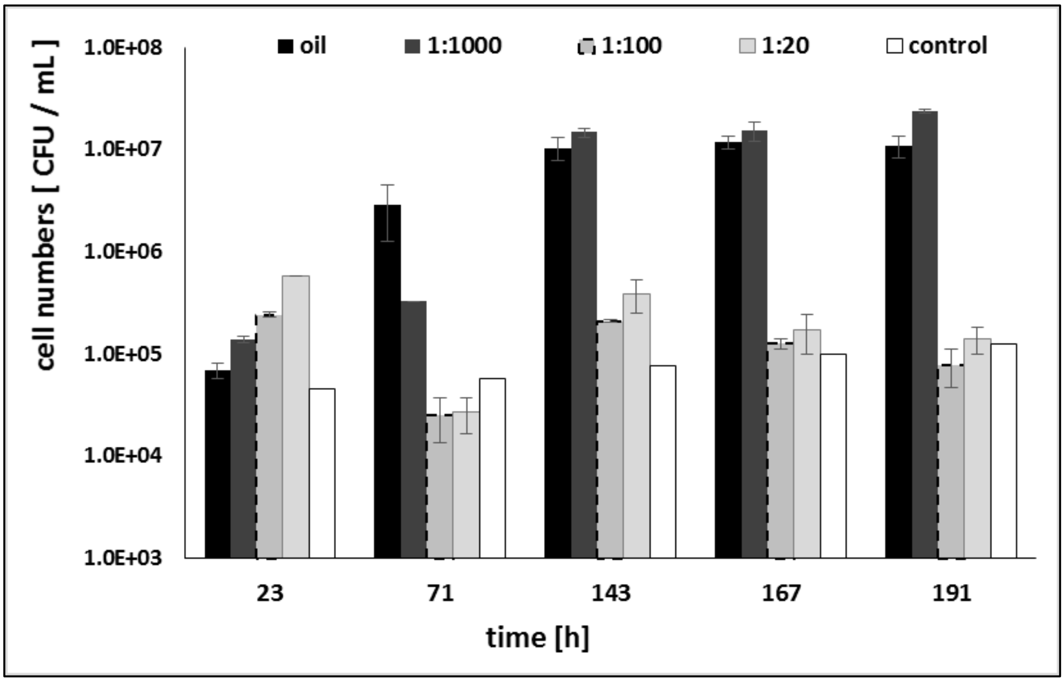


Fig. 3.11: Cell concentrations [CFU mL^{-1}] of Rhodococcus *sp. PC20 in 20 mL MM2 with 200 µL crude oil and varying concentrations of dispersant (0, 0.2, 2, 10 µL) at 0.1 MPa, RT, 200 rpm. n = 2.*

With a low ratio of dispersant to oil (DOR 1:1000), cell numbers of *Rhodococcus* sp. PC20 increased, after an initial delay to enter the exponential growth phase (Fig. 3.9 – Fig. 3.11), in comparison to the crude oil only incubation. A slightly, but not significant, higher maximum cell concentration with dispersed oil compared to the crude oil only incubation might be an effect of the higher dissolution rate of substrate caused by the dispersant.

In contrast to this, higher ratios of dispersant to crude oil resulted in significantly reduced growth, below the CFU concentration of the no substrate control (Fig. 3.10 / Fig. 3.11). Generally, when resulting CFU concentrations were lower than those of the no substrate control, the treatment was considered toxic to *Rhodococcus* sp. PC20. This was the case in the incubations with DORs of 1:20 and 1:100 at several time points.

Dispersant produces a smaller droplet size emulsion of crude oil in seawater. After finding the ratio, at which dispersant becomes toxic to *Rhodococcus* sp. PC20, it was investigated whether this observation was initiated by toxic compounds originating from the dispersant or the higher dissolution from the crude oil, initiated by the increased surface area of the crude oil. A higher surface area causes an increase in mass transfer rates of potentially toxic compounds from the organic to the aqueous phase. *Rhodococcus* sp. PC20 was able to grow on LB medium.

This is why LB was chosen as a second complex substrate to investigate effects of dispersant on the strain (Fig. 3.12).

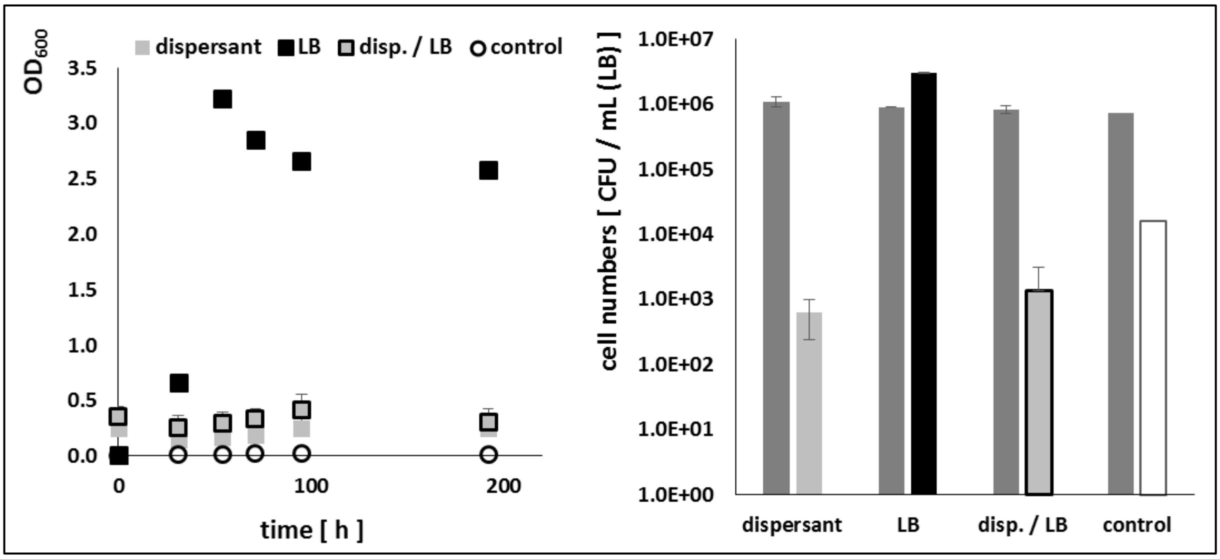


Fig. 3.12: left: Photometrical measurements of Rhodococcus *sp. PC20 in 20 mL MM2, with combinations of LB (150 mg) and dispersant (2 µL) over 192 h at 0.1 MPa, RT, 200 rpm. n = 2. right: cell concentrations [CFU mL^{-1}] at 0 h and 95 h, in 20 mL MM2 with LB (150 mg) and dispersant (2 µL) at 0.1 MPa, RT, 200 rpm.*

With Lysogenic Broth (LB), it was possible to measure growth of *Rhodococcus* sp. PC20 photometrically. This is more reliable in comparison to incubations with crude oil, as LB does not emulsify in the medium. Substantial growth (OD > 2.0) could only be detected when the substrate (LB) was added without any dispersant to the incubation. OD values of all incubations where dispersant was added stayed below 0.5 over the whole incubation time.

Accordingly, results of the CFU measurements revealed a decrease in cell numbers, when dispersant was present in the incubation. Therefore, dispersant can be considered toxic towards *Rhodococcus* sp. PC20 with concentrations higher than 0.01 % v/v of dispersant in the medium (equivalent to a DOR of 1:100).

3.1.5. Growth of *Rhodococcus* sp. PC20 Under Deep Sea Pressure Conditions and With the Addition of Chemically Dispersed Crude Oil

Combining the findings of the previous chapters, the combination of pressure and dispersant were investigated to answer, whether dispersant has the same effect on *Rhodococcus* sp. PC20 at deep sea pressure conditions (15 MPa).

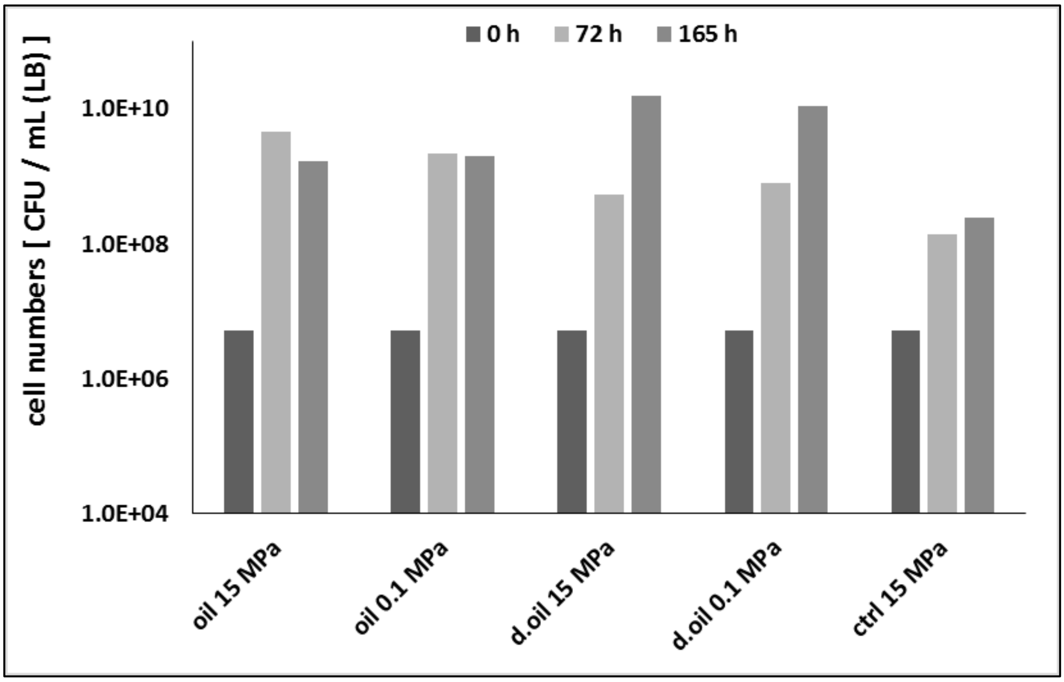


Fig. 3.13: Cell concentrations [CFU mL^{-1}] of Rhodococcus *sp. PC20 in 20 mL MM2, with crude oil (200 µL) and dispersed oil (0.2 µL dispersant additionally (**1:1000**)) over 165 h at 0.1 / 15 MPa, RT, 200 rpm. n = 1. Oil – crude oil, d.oil – dispersed oil, ctrl – control.*

Similar to the incubations at atmospheric pressure, the addition of small amounts of dispersant (DOR 1:1000; Fig. 3.13) did not show any inhibiting effect on the growth of *Rhodococcus* sp. PC20 at elevated pressure. Even an increase in the cell numbers (crude oil only: 1.8 ± 0.2*10^{9} CFU mL^{-1}; dispersed crude oil: 1.4 ± 0.3*10^{10} CFU mL^{-1}) was detected after the incubation for 165 h compared to the crude oil incubations. As it has already been discussed in chapter 3.1.3 for the oil only incubation, pressure did not play a significant role regarding cell concentrations.

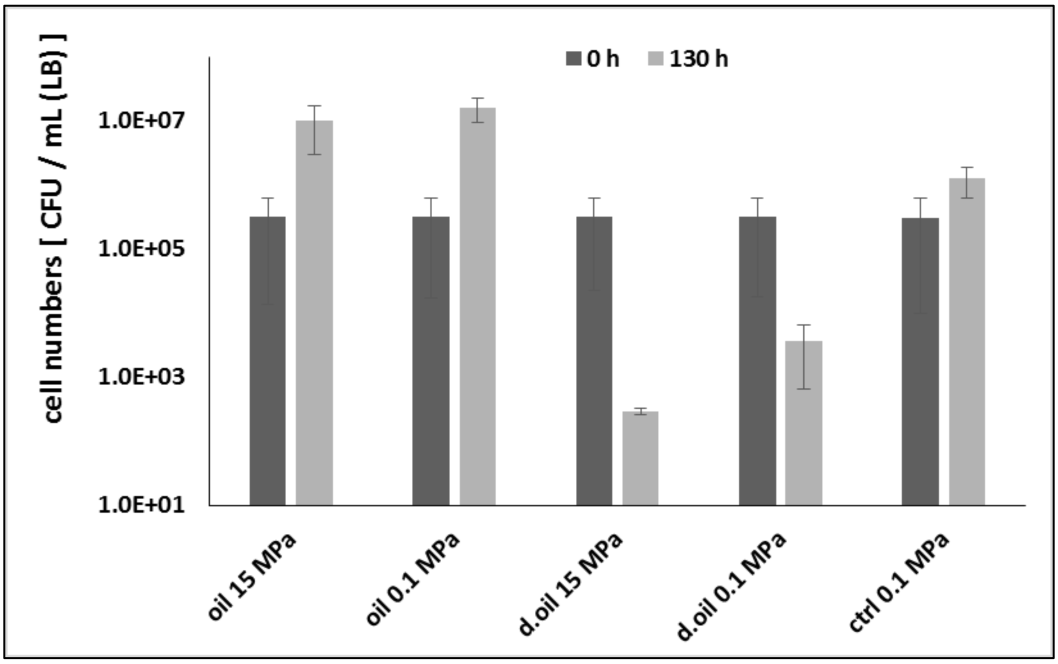


Fig. 3.14: Cell numbers [CFU mL^{-1}] of Rhodococcus *sp. PC20 in 20 mL MM2, with crude oil (200 µL) and dispersed oil (2 µL dispersant additionally (**1:100**)) over 130 h at 0.1 / 15 MPa, RT, 200 rpm. n = 2. Oil – crude oil, d.oil – dispersed oil, ctrl – control.*

Applying more dispersant (DOR of 1:100) to the incubation at elevated pressure led to a decrease in cell numbers after 130 h (Fig. 3.14). These findings suggest that pressure enhanced the effect of dispersant. There was a significant difference between the cell numbers at atmospheric and elevated pressure of the dispersed crude oil incubations (Wilcoxon Rank Sum test). The specific amount at which dispersant addition (DOR 1:100) led to inhibition in growth of *Rhodococcus* sp. PC20 did not change at elevated pressure. At elevated pressure, the addition of dispersed oil (DOR 1:100) resulted in even smaller cell numbers compared to the atmospheric pressure incubation (Fig. 3.14). Pressure and dispersant might have a negative synergistic effect on *Rhodococcus* sp. PC20's growth.

***Rhodococcus* sp. PC20 is able to metabolize aliphatic hydrocarbon rather rapidly without a sensitivity towards any pressure from surface to deep sea conditions. The addition of dispersant in the aftermath of the DWH incident might have prevented the indigenous *Rhodococcus* species from becoming relevant in plume and sediment communities. Dispersant might have been a selection factor in the communities responding to the hydrocarbon input.**

3.1.6. Error Analysis

Pipetting small amounts of substrate (oil, dispersant) might have led to variations in the carbon and energy supply to the incubated cultures. For a more reproducible way of quantitatively adding crude oil to the incubation a glass syringe (1 mL, 1000 Series, Hamilton Bonaduz AG, Bonaduz, Switzerland) was used. Dispersant was added in small amounts (0.5 – 2 µL) to the incubations. Pipetting those amounts could have led to a systematic error of 0.04 – 0.50 µL (manufacturer information). The expected error might be higher as dispersant (Corexit EC9500A) is comparably more viscous (70.0 mm^2/s (15.6 °C), manufacturers information) than water (1.1 mm^2/s (16.0 °C). The CFU method is susceptible to errors by variations in the agar medium provided. Precultures were prepared with a single loop of grown culture, while medium and incubation times were kept constant. Even though the status of the organisms (alive, active, dead) influenced the starting CFU concentration in the subsequent experiments. A direct measurement before the incubation start was not possible, as the direct measurement of the optical density was highly influenced by crude oil droplets. The parallel incubation of a biotic control (no substrate) was necessary to enable a comparison in each experiment. Oxygen measurements resulted in high standard deviations (Fig. 3.8), which originate from numerous sources. Besides the pipetting errors, the starting concentrations of the inoculum (CFU / mL) varied to a greater extend. Further, it took the oxygen sensor (PreSens) a minimum of 5 hours to stabilize after pressurization to 10 / 15 MPa.

3.2. Response of Northern GoM Bacterial Surface Sediment Community to Crude Oil, Dispersant and Pressure

The majority of crude oil compounds introduced to the northern GoM after the DWH incident was biodegraded by the indigenous microbial community (Joye, 2015). A variety of bacteria, already known, as well as so far unknown crude oil degraders, responded in successive stages to the massive input of hydrocarbons and dispersant (King et al., 2015). The metabolic potential of the microbial community exceeds the limited ranges of single crude oil degraders. Information on the responses of the GoM deep sea microbial communities to environmental factors, like pressure and dispersant, are of great importance in modeling deep sea oil spill scenarios.

We were supplied with different sediment samples from the northern Gulf of Mexico from the vicinity of the exploded well (Tab. 2.4) by our cooperation partners in the C-Image II consortium. The rational for incubation of the upper layer of sediment was to incubate those strains recently settled to the surface layer from the water column. The volumetric cell numbers (per cm^3) of the sediments are higher than in the overlying water column (Orcutt et al., 2011) and were supposed to yield higher activities.

Aim of this study was to evaluate the responses of the community from the GoM deep sea sediment layer especially to the environmental factors pressure, dispersant and crude oil in *ex situ* experiments. In contrast to the previous chapter, the rational of incubating the sediments at 10 MPa is the pressure representing the water column depth at which the hydrocarbon plume formed after the DWH incident (1000 -1200 m, Camilli et al., 2010).

The conducted experiments, which included a subsequent 16S rRNA sequence analysis of the microbial community, are listed in Tab. 2.5. Preliminary experiments were conducted to investigate the reproducibility of experiments (**3.2.1**), absolute values of cell numbers of the incubations (**3.2.2**), and the effect of incubation media and incubation times (**3.2.3**).

With the lessons learned from these preliminary experiments, the main experiments were designed and analyzed (**3.2.4**). Subsequently the statistical evaluations with focus on the influence of environmental factors like pressure (**3.2.5**) and substrate (**3.2.6**) on the bacterial community are discussed. Finally, a model, which lays weight on multiple parameters, is evaluated (**3.2.7**).

3.2.1. Reproducibility of Experimental Runs

Deep sea sediments are very heterogeneous environments (Orcutt et al., 2011). The sediments, which were used as inoculum for the experiments, were sampled from the Gulf of Mexico and shipped to Hamburg, where they were stored at 4 °C until they were used in the experiments. In order to obtain a representative northern GoM deep sea sediment sample, five sediments were thoroughly premixed in equal amounts before aliquoting them to the reaction vials. There was a great variability of the samples with sequence read counts between $3.92*10^3$ and $2.11*10^5$ (average: $5.33*10^4$) per sample belonging to 177 identified taxonomic genera (average per sample). As samples were mixed from five different sediment samples, it was of interest, whether a homogeneous and reproducible initial community could be provided by the procedure (t_0, Fig. 3.15).

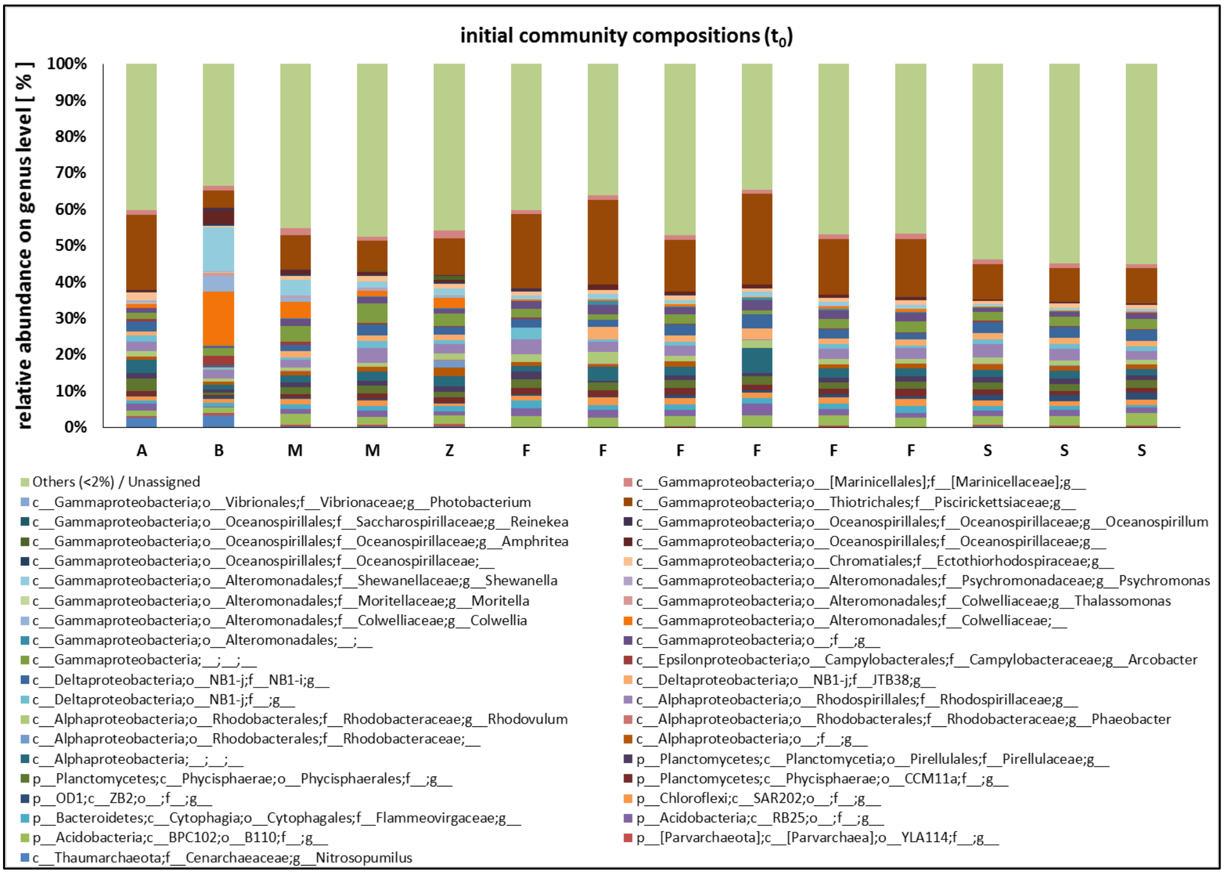


Fig. 3.15: Microbial community relative abundance of all initial time points different experiments on genus level. ASW and FSW, 4 °C. A – Exp. 10, B – Exp. 12, M – Exp. 13, Z – Exp. 15, F – Exp. 17, S – Exp. 16 from Tab. 2.5. Multiple letters represent repetition of the respective experiment.

Factors like used sediment volume, storage time, sampling bias, number of successful Ilumina sequence reads might have been leading to the observed variations within the initial community composition.

To put these variations into perspective, the distances between all conducted samples (principal coordinate analysis, PCoA, Bray Curtis distance matrix), i.e. differences of the samples in respect to their relative abundances were investigated. This helped to easily distinguish between the t_0 samples and all remaining incubations (Fig. 3.16), as t_0 samples clearly formed a group. Further, samples tended to form groups among experiments (e.g. Exp. Oct16), but are not clearly separated. Overall, no general pattern can be implied, as samples were widely scattered and neither clearly grouped by experiment nor treatment.

Statistical test on all conducted samples (PERMANOVA, 999 permutations, p = 0.001, n = 125, Anderson, 2001) showed significant differences comparing incubations with a run time for more than a day (7 – 56 days, n = 115) with the initial incubation point (t_0, n = 14) samples.

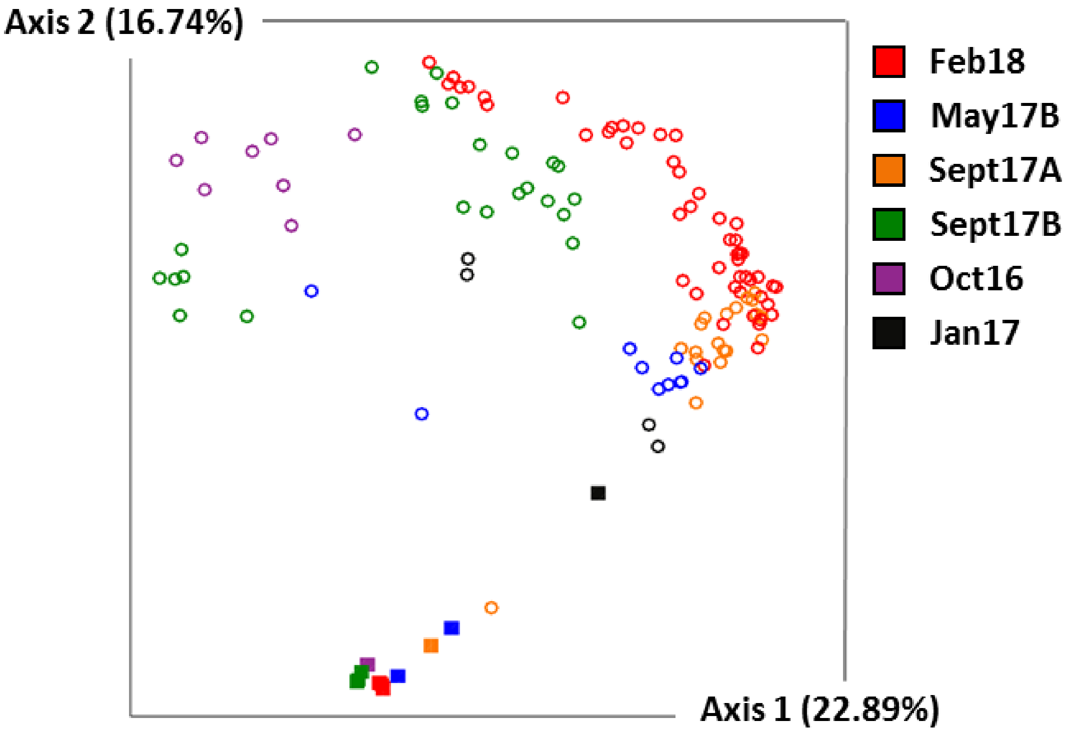


Fig. 3.16: PCoA plot (Bray Curtis dissimilarities) of all experiments (color-coded). Open circles indicate incubations for t > 1 day and closed squares for time zero.

Further pairwise comparison of whole experiments (and not each sample) to each other, revealed that each experiment is varying significantly from each other ($p < 0.002$, further information in Suppl.Tab.1).

The diversity of the microbial community at the initial incubation point (t_0) showed an evenly distributed community (Pielou's Eveness index: 0.92 ± 0.01 (n = 9) / observed OTUs: 1172 ± 698 (n = 9)). Proteobacteria (Alpha- [9.8 ± 2.0 %], Delta- [12.3 ± 1.4 %] and Gamma- [30.8 ± 5.7 %]) dominated the initial community, together with Phycisphaerea [7.0 ± 1.0 %] and in lower abundances Thaumarcheota [0.5 ± 1.0 %], belonging to the Archaea.

Generally, incubation at any pressure (0.1 / 10 MPa), any substrate (none, crude oil, dispersant, dispersed oil) and for any incubation time (7 – 56 days) led to a drastic drop in diversity (Pielou's Eveness index: 0.65 ± 0.06 (n = 35) observed OTUs: 453 ± 257 (n = 35)). Gammaproteobacteria became dominant [79.8 ± 7.8 %] in the microbial community at the cost of all other classes, which decreased in relative abundance (e.g. Alphaproteobacteria [4.3 ± 2.5 %]). Within the Gammaproteobacteria the order of Alteromondales was increasing in relative abundance of the whole community [t_0: 6.0 ± 2.9_%, $t_{7\text{-}56d}$: 73.5 ± 18.0 %] with any incubation.

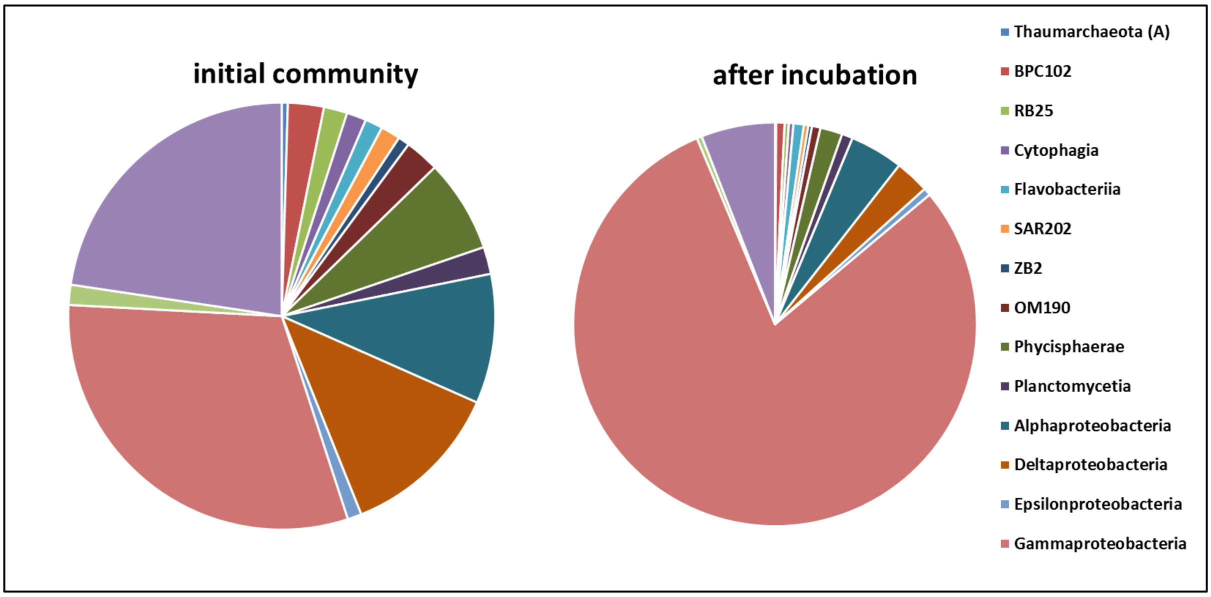


Fig. 3.17: Averaged microbial community structure (class) before (n = 12, left) and after (n = 35, right) incubation (without substrate, 0.1 and 10 MPa, 7 – 56 days) of all experiments (Tab. 2.5).

The microbial diversity of the initial communities represent a typical oxic surface deep sea sediment community (Fig. 3.17; Orcutt et al. 2011). Especially, Alteromondales (Colwelliaceae, Moritellaceae, Shewanellaceae) and Oceanospirillaceae were able to increase their relative abundance during incubations. All these families are common representatives of the pelagic deep sea (Orcutt et al. 2011, Lopez-Lopez et al. 2005, Zaballos 2006). While Yang et al. (2016) found far less gammaproteobacterial dominance in any surface sediment investigated around the DWH well, between May 2010 and July 2011.

These changes from a typical sedimentary prokaryotic community occur without the addition of any substrate and (on a family level) without any differences of the pressure applied. Therefore, those changes are induced by the parameters of our setup and have to be considered in interpretation of all following experiments and analysis. There is a rapid change from the merely pristine, natural setup of the sediment (either in the deep sea or in the sampling jar) with limited diffusion, and therefore limited accessibility of nutrients in well-established micro environments. Due to the stirred incubation, where fresh seawater is added, a transition from a typical sediment community to a deep sea pelagic community was observed here.

Overall changes in the community composition were detected with every incubation step within every experiment and across experiments. It is impossible to elucidate the reason for the variability, which was detected already among the t_0 samples. Whether these differences are originating from real differences in the prokaryotic community structure or

are caused by sampling biases, was not of interest to be answered in this study. Therefore, every experiment was performed including a biotic control. Thereby the influences of the setup can be deducted for each experiment.

3.2.2. Absolute Quantification of Cell Numbers From Incubation Experiments

The information derived from sequencing and downstream analysis is a relative proportion of the community, depending on the successful reads in each sample. To obtain quantitative data on the experiments, cell count measurements (CFU) were conducted in parallel on marine agar (MA) to obtain information on the absolute abundance.

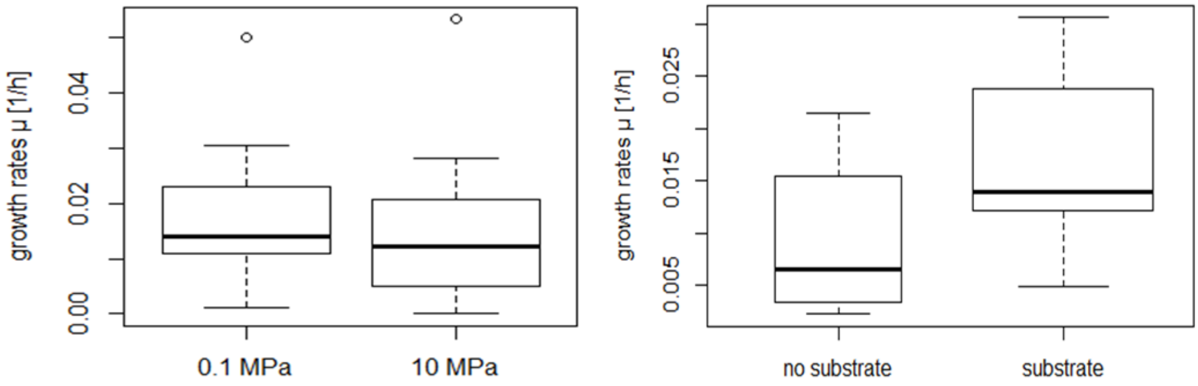


Fig. 3.18: Growth rates [h^{-1}] boxplots of the cultivable bacteria (on MA agar) of all experiments. Shown for different pressure categories (0.1 / 10 MPa, left) and for the substrate categories (right).

With the exception of one, all experiments (n = 9) with sediment as inoculum showed growth of the sediment microbial community in comparison to the starting concentration.

All samples, where CFU concentrations could be evaluated (n = 41), showed growth rates between -0.004 and 0.053 h^{-1} with a median of 0.010 h^{-1} with experiments run between 92 and 792 hours (Fig. 3.18). Negative growth rates represent higher cell death rates than growth rates.

All incubations with substrate added (oil, oil/methane, dispersant, dispersant/oil, dispersant/oil/methane) showed significant differences in growth compared to experiments, where no substrate was added (Fig. 3.18). Comparing all incubations conducted at 0.1 MPa with those grown at 10 MPa revealed no significant different growth rates between them.

The average of all measured CFU concentrations at t_0 was at 4.1 ± 1.7 x 10^3 CFU mL^{-1} (n = 15), while all incubations regardless of pressure or substrate for 168 h resulted in 4.4 ± 5.9 x 10^4 CFU mL^{-1} (n = 53). These numbers ideally fall into the average range of deep sea pelagic prokaryotic cells (10^3 to 10^5 cells mL^{-1}) in the deep sea (Nagata et al., 2000, Reinthaler, 2006) and the number of cells detected in the plume (5.5 × 10^4 cells cm^{-3}) and outside the plume (2.7 × 10^4 cells cm^{-3}) (Hazen et al., 2010).

The reason, why these numbers have to be interpreted with caution, is because of the method they were obtained with. The CFU method may overestimate a subset of species or even just a single species, which is selected for by the conditions on the plate and discriminates against species with varying growth requirements. Regarding those selected organism(s), a significant growth on MA plates was shown.

3.2.3. Influence of Incubation Time and Media on the Bacterial Community

Time The bacterial community in the plume did undergo drastic changes, even after the well was capped (King et al., 2015). Driving force for the detected succession of dominant species was the availability of hydrocarbons, which in turn was influenced by multiple factors (temperature, nutrients, and circulation, Dubinsky et al., 2013). Therefore, the time to sample and to evaluate the bacterial community is a crucial parameter.

This was addressed from two points of view. The first was to find the earliest time-point after the community changed to a relatively static composition without any substrate influences. The other approach was to see whether first data from the field (Hazen et al., 2010) could be replicated in *ex situ* experiments.

Starting with the first approach, the bacterial community changed drastically within the first seven days of incubation. Five time points (t = 0, 1, 3, 6, 7) were investigated to track the initial transitions (Exp. Sept17A, Fig. 3.19).

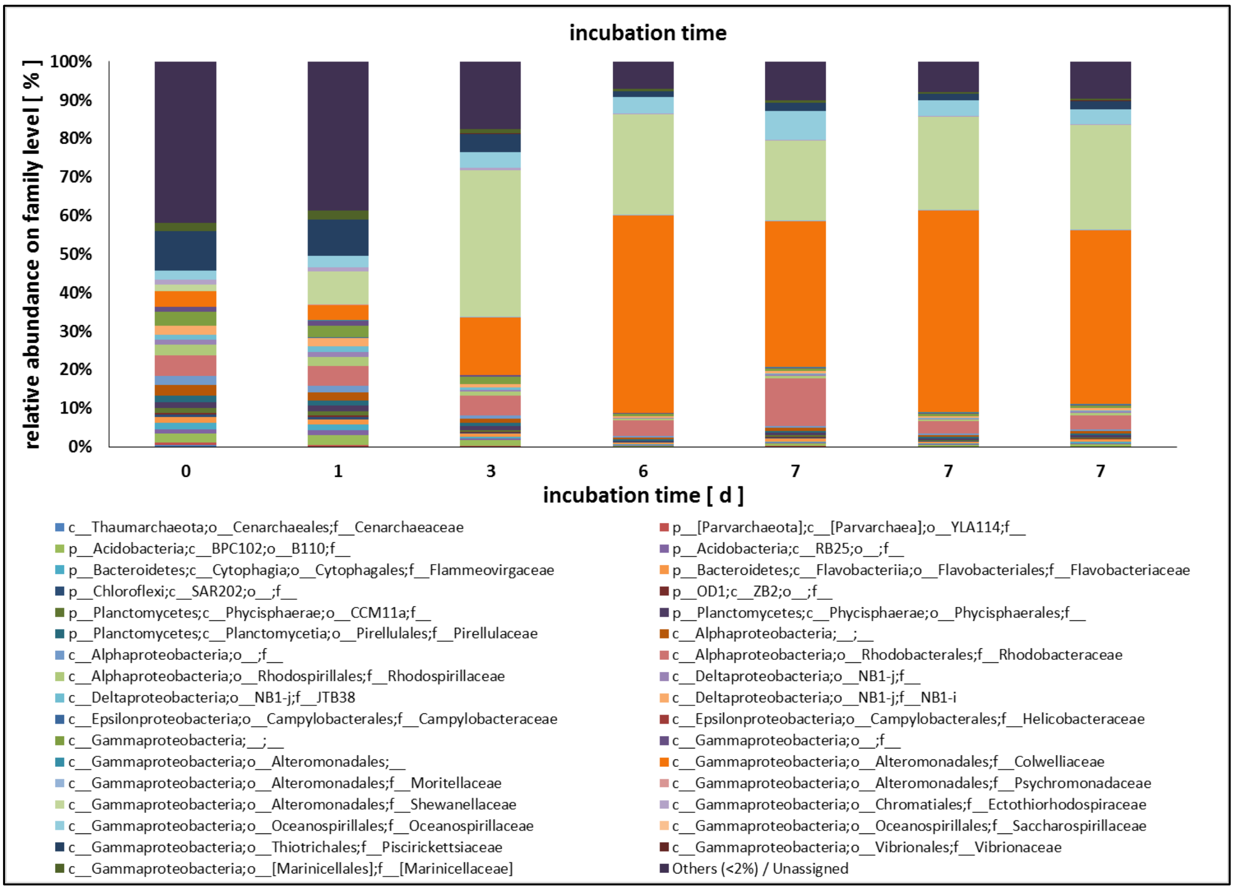


Fig. 3.19: Relative abundances on family level of one experiment (Sept17A) without any substrate for 0, 1, 3, 6 and 7 (3 repetitions) days in filtered seawater (FSW) at atmospheric (0.1 MPa) pressure and at 4 °C. Multiple bars with the same incubation time represent repetitions.

For this experiment, no substrate and pressure were applied and the sediment mixture was incubated with filtered seawater (FSW) at 4 °C. Obviously, the community composition started to change already after 36 hours and kept changing until seven days (Fig. 3.19).

To put these initial changes into perspective, experiments were combined, with those that were run for a longer period (> 7 days; experiment Oct16, Jan17, May17A, Fig. 3.20). All these experiments were conducted without substrate addition in ASW and at elevated pressure. The different initial conditions of the experiments evaluated together have to be considered. However, these investigations were conducted to see general changes in the stability of the communities.

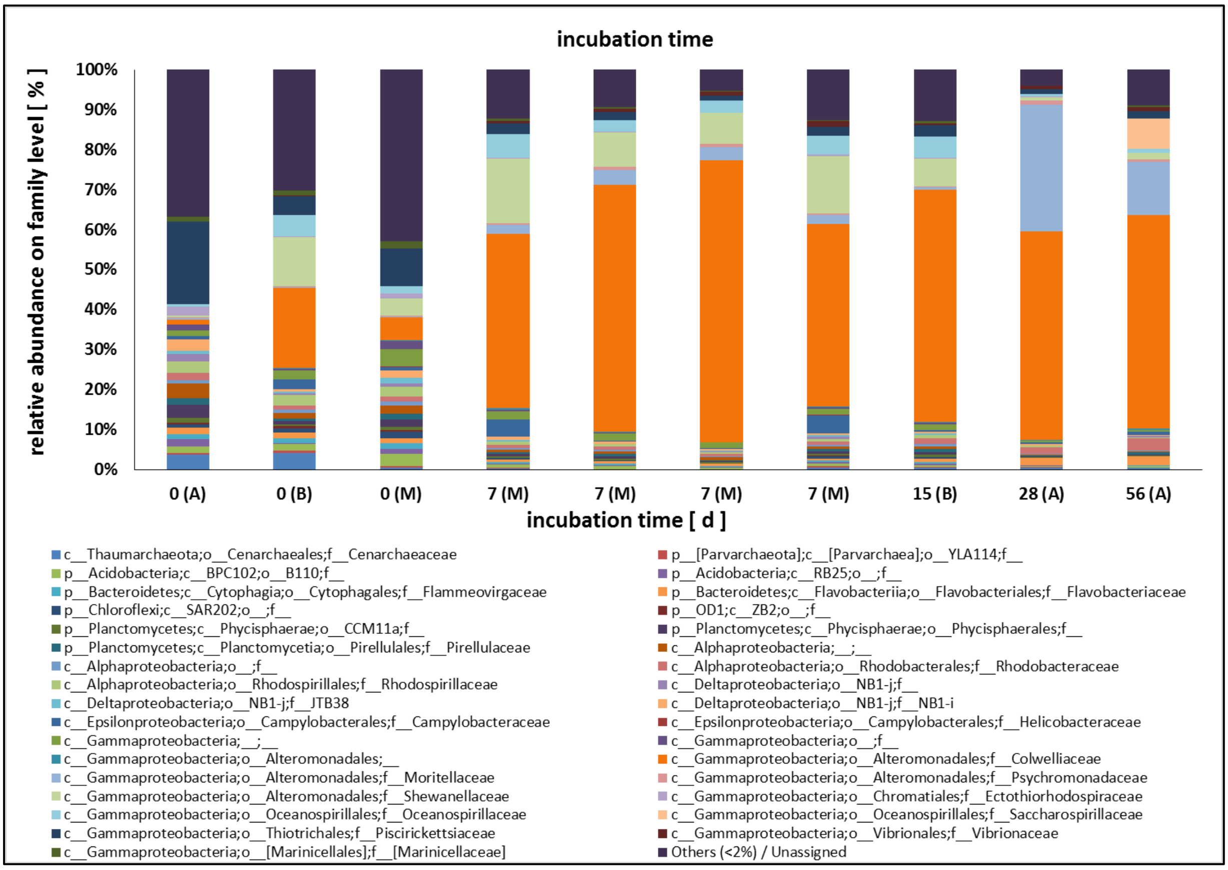


Fig. 3.20: Relative abundances on family level from three experiments (A – Oct16/Exp.10, B – Jan17/Exp.12, M – May17A/Exp.13, from Tab 2.5) without substrate added for 7, 15, 28, 56 days in artificial seawater (ASW) at elevated (10 MPa) pressure. Multiple bars with the same incubation time represent repetitions of the respective experiment.

Apparently, the changes within seven days in the microbial community were more drastic than the changes thereafter (15, 28, 56 days). On a family level, Colweilliaceae dominated any incubation under the given condition (no substrate, ambient pressure, ASW / FSW) over the observed time span (average t_0: 9.0 ± 8.0 %, average $t_{7\text{-}56}$: 55.1 ± 8.7 %). Besides this, the community kept on changing to some extent. For example, Moritellaceae gained in relative abundance in the longer incubations (t_7: 2.8 ± 0.6 %, t_{28}: 31.8 %, t_{56}: 13.3 %), while Shewanellaceae was found less in the longer incubation compared to the incubation for one week (t_7: 11.7 ± 3.7 %, t_{28}: 1.0 %, t_{56}: 1.7 %). One Oceanospirillales family, Saccharospirillaceae, has not been detected until the latest incubation point (average t_0-t_{28}: 0.02 ± 0.03 %, t_{56}: 7.7 %). A similar change in the community composition and the same overall outcome was observed at atmospheric pressure (Suppl.Fig.1).

Statistical analysis of the bacterial communities (Kruskal Wallis Test) revealed significant differences only after day 7, when compared to the initial community compositions (Tab. 3.1).

The diversity indices showed a similar pattern, as the diversity indices stayed merely constant after seven days (Fig. 3.21).

Group 1	Group 2	H	p-value
0 (n=11)	1 (n=1)	1.7	0.192
0 (n=11)	3 (n=1)	2.5	0.111
0 (n=11)	6 (n=1)	2.5	0.111
0 (n=11)	7 (n=99)	29.4	**0.000**
0 (n=11)	15 (n=4)	8.3	**0.004**
0 (n=11)	28 (n=4)	8.3	**0.004**
0 (n=11)	56 (n=4)	8.3	**0.004**

Tab. 3.1: Kruskal Wallis Test to investigate significant differences between all incubations times (1 – 56 days), each compared to the initial point (t_0). Significance levels < 0.05 in bold.

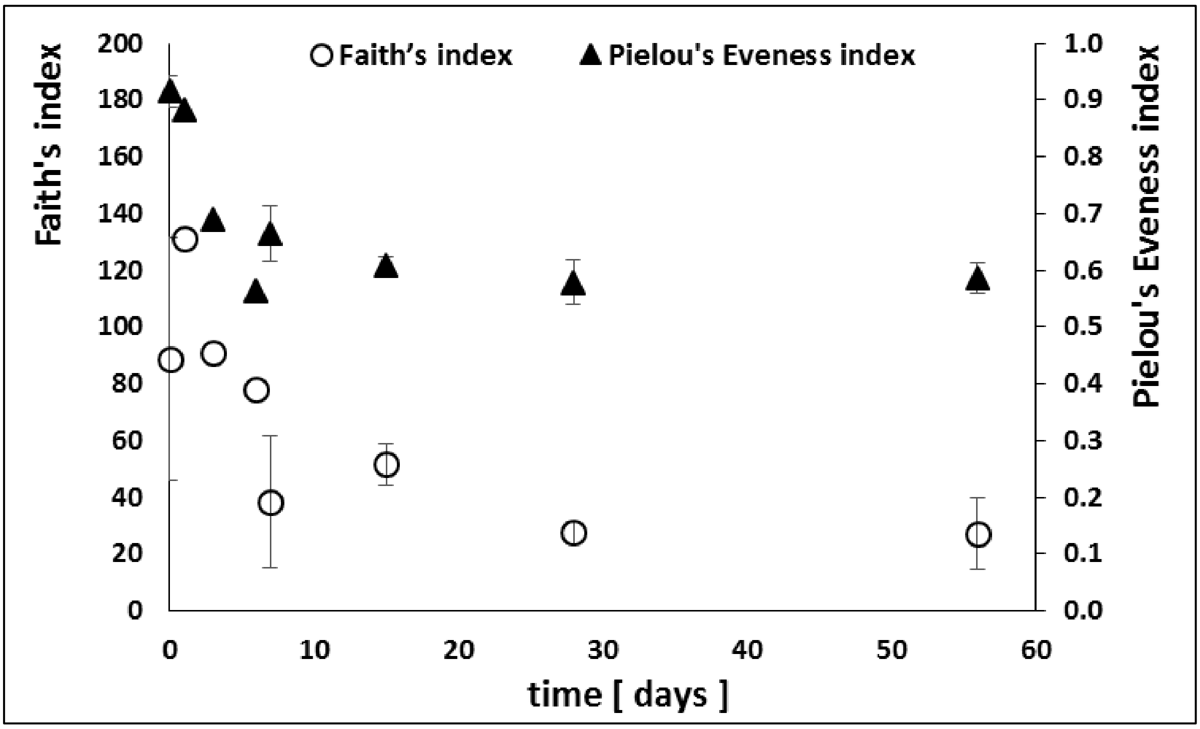


Fig. 3.21: Faith's Phylogenetic Diversity (community richness, incorporates phylogenetic relationships between the features) and Pielou's Evenness (community evenness) of all experiments over incubation time (0 – 56 days).

The bacterial community was constantly changing. The decision for a seven day incubation in all subsequent experiments was made for the following reasons: a significant change in

the bacterial community composition was detectable after seven days (Tab. 3.1) and a longer incubation time (> 7 days) did not lead to an additional major drop in the diversity indices (Fig. 3.21). A relatively short incubation time was also advantageous because more experiments were possible within a given time.

Addressing the second approach, first *in situ* data (May, 2010, Hazen et al., 2010) on the bacterial community of the plume were available one month after the DWH oil spill. Incubation times were adjusted to match this time span (28 and 56 days) to evaluate how comparable the data in this study are to *in situ* data. The experiment to compare with the data from Hazen et al. (2010) was conducted with ASW, 1 % crude oil, at 10 MPa and additional 1 MPa of methane, which resembled the natural gas leaked among the hydrocarbons in the uncontrolled oil flow phase.

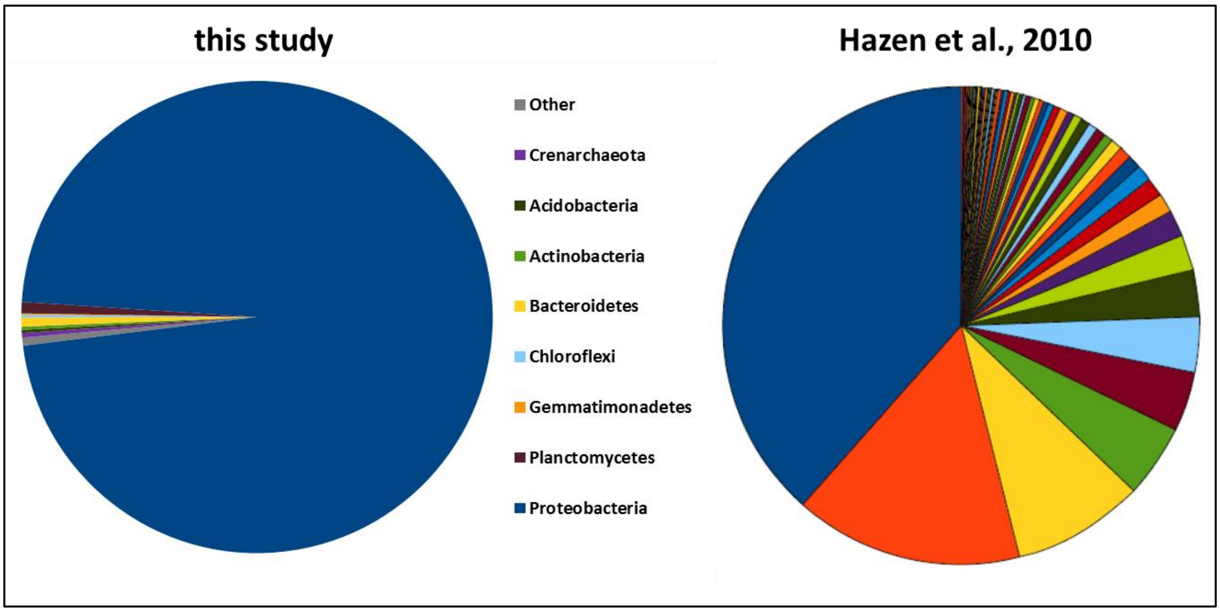


Fig. 3.22: Comparison of incubation for 28 days (ASW, 10 MPa total pressure, 1 MPa methane, n = 1) from this study (left) with published data from the plume 35 days after the DWH explosion (right, Hazen et al., 2010). Only phyla of > 1 % relative abundance and which were found in both studies are shown, except for Crenarchaeota (Archaea) and the category "Other" is not present in the right pie chart, as all abundant phyla are displayed.

A comparison with the data from Hazen et al. (2010) (Fig. 3.22) showed that similar dominant phyla (Proteobacteria, Bacteroidetes) were found, but poorly matched the proportions of the whole community.

These differences in comparison to *in situ* conditions may originate from the artificial setup, the varying sequencing methods, and/or the different origin of the inoculum. It is important to mention, that the aim of this thesis was not to mimic the conditions of the deep sea, but to focus on the effects on the bacterial community in a closed system with a confined parameter window (pressure, substrates).

Medium Marine salt and trace element concentrations, especially of the open oceans, are globally quite uniformly distributed (Castro and Huber, 1997). This is why artificial seawater (ASW) was widely used in laboratory experiments simulating ocean waters. The requirements of the bacterial metabolism for nutrients and trace elements are often highly specific (Morel and Price, 2003) and a depletion in nitrogen and phosphate with hydrocarbon surplus may lead to a limitation in growth.

Two types of media, resembling seawater conditions, were taken into consideration: The artificial seawater (ASW) and filtered seawater from the northern Gulf of Mexico (FSW).

The same sediment inoculum (Fig. 3.23; Sept17A) was incubated in the two media under the same pressure (0.1 / 10 MPa) and substrate (crude oil / no substrate added) conditions. Statistical analysis (Wilcoxon Rank sum test, 2-sided, $\alpha = 0.05$, $n = 4$ (ASW) and 12 (FSW)) of the 14 genera with relative abundances above 2 % did not reveal any significant differences among the two groups. Samples were divided into the incubation medium categories regardless of pressure and substrate.

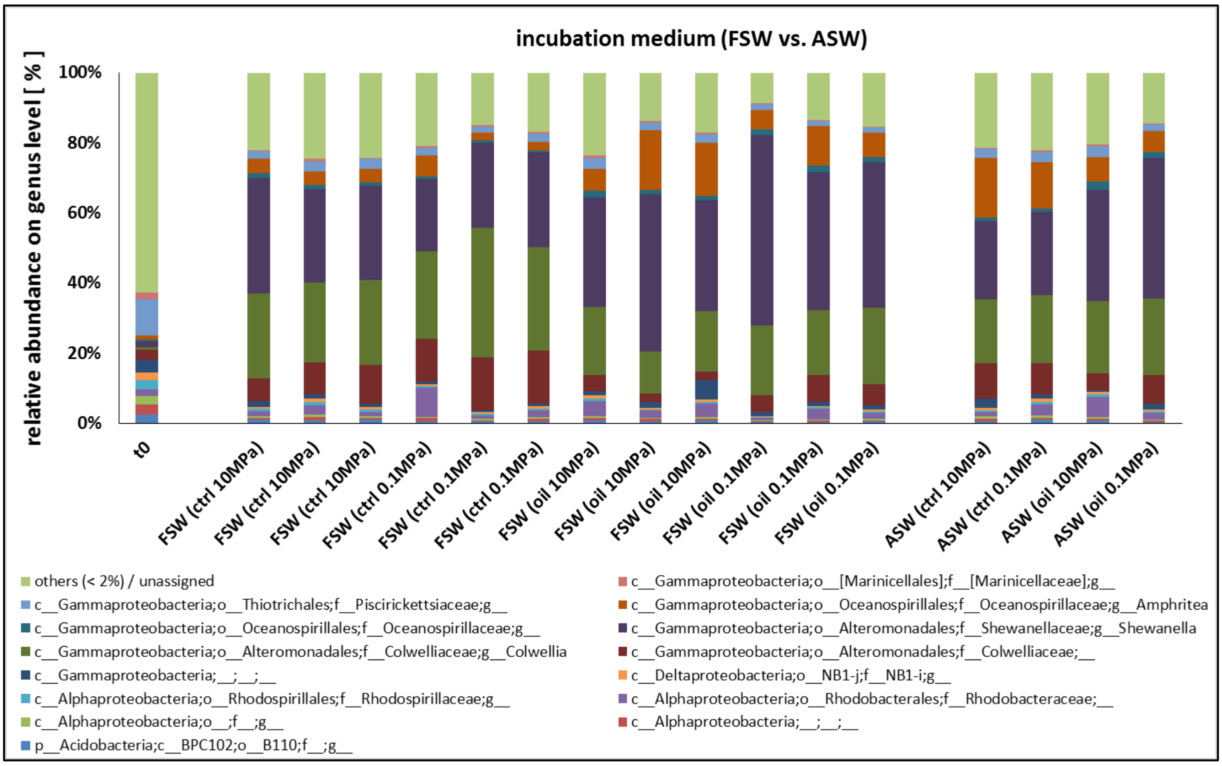


Fig. 3.23: Relative abundances [%] on genus level from one experiment (Sept17A, Exp. 15, Tab. 2.5) no substrate and crude oil (1 % v/v) for 7 days in filtered (FSW) and artificial seawater (ASW) at atmospheric (0.1 MPa) / elevated (10 MPa) pressure. oil – crude oil, ctrl – no substrate added.

There were not sufficient sample size to distinguish between categories considering medium, substrate and pressure. By splitting the sample data into categories of incubation pressure and substrate, differences in single genera were more pronounced (Fig. 3.24), but a sample size of one (ASW) and three (FSW) is too small to apply statistical analysis.

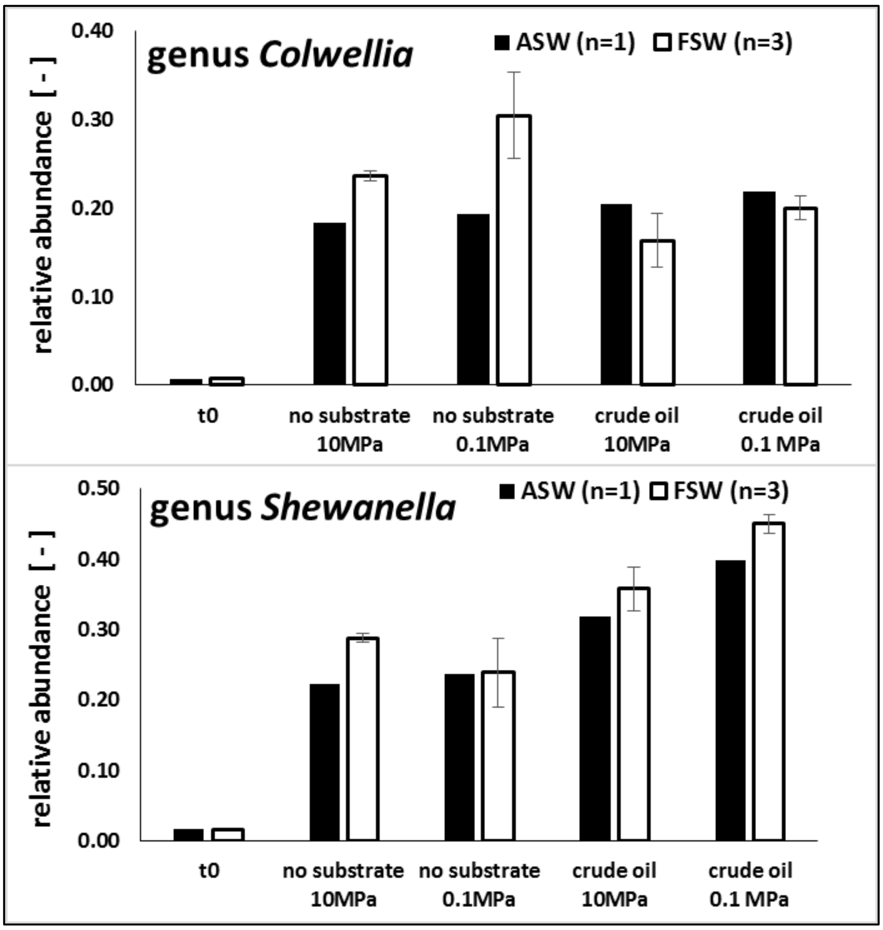


Fig. 3.24: Relative abundance [-] of the genera Colwellia *and* Shewanella *of all incubations (Sept17A, Exp. 15, Tab. 2.5) by substrate and pressure categories. 20 mL medium (ASW / FSW), 200 µL crude oil (1 % v/v), 4°C, 168 h, n = 3, 200 rpm.*

***Ex situ* experiments are vulnerable to biases by the incubation setup. Therefore, the choice of the incubation medium is critical to the interpretation of experiments. However, as there are just minor differences in the community composition in different media, the medium closer to *in situ* conditions (FSW) was chosen for conducting the main analysis on the influences of pressure, oil and dispersant on the bacterial community.**

3.2.4. General Remarks on the Main Experiments (Sept17B and Feb18)

All considerations of the preliminary experiments (chapters 3.2.1 – 3.2.3) led to the design of the two major experiments. Experiment Feb18 and Sept17B were incubated with sufficient repetitions (n = 6) with and without substrate (oil, dispersant) and at both pressures (0.1 / 10 MPa) for statistical analysis, but without a control at 0.1 MPa in the Sept17B experiment. Also no incubation with dispersant as only carbon source was added in the Sept17B experiment.

Both experiments were incubated with 250 mg of sediment mixture in 5 mL of filtered seawater (FSW) for seven days.

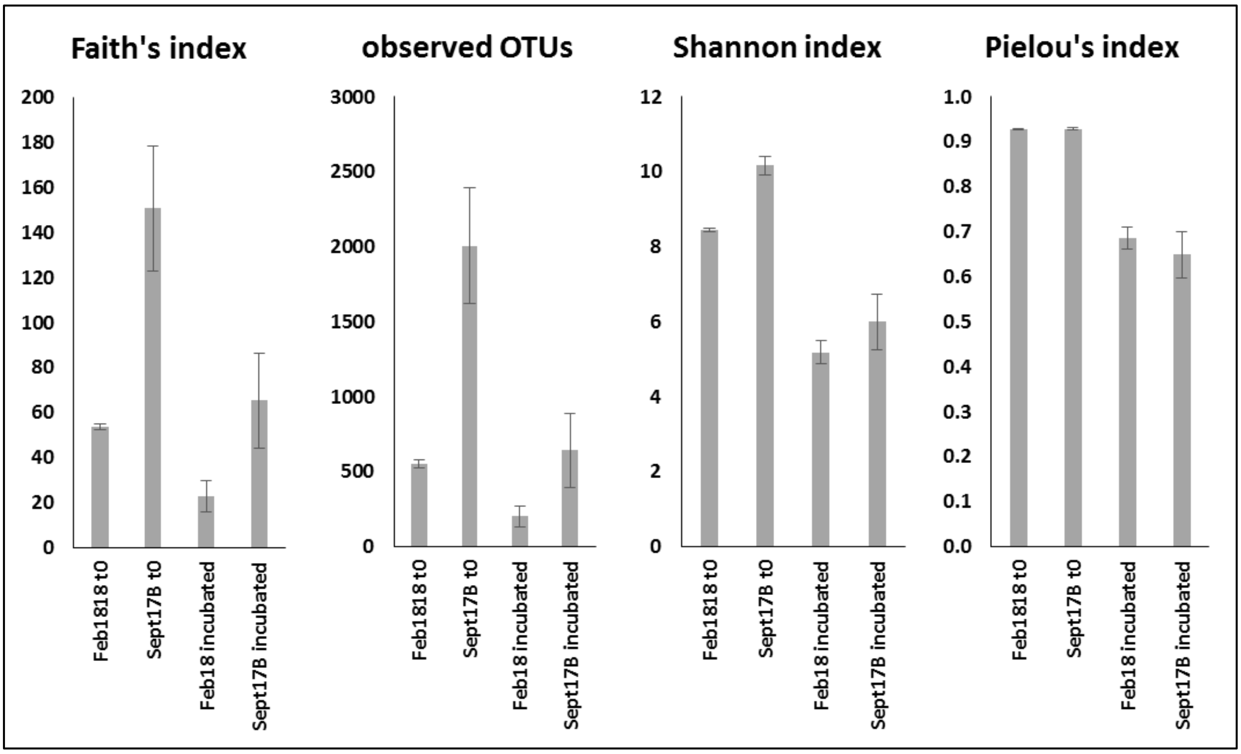


Fig. 3.25: *Diversity indices (Faith index, observed OTUs, Shannon index and Pielou's Eveness index (Pielou)) for each experiments (Sept17B and Feb18, Exp. 16 and Exp. 17, Tab. 2.5) initial community (t_0) and all other incubations (t > 0).* 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4 °C, 168 h, n = 5 – 6, 200 rpm.

Diversity indices calculated for the two separately conducted experiments (Sept17B and Feb18) showed similar community evenness (Pielou's eveness index), but deviations among the community richness (Fig. 3.25; Sept17B > Feb18). As a consequence, statistical analysis (PERMANOVA, Bray Curtis dissimilarities, p = 0.001, 999 permutations) also found significant differences between the two experiments. This showed again, that these experiments resulted in a different outcome. These separate analysis outcomes were compared. Analyzing the differences among samples within each experiment (Fig. 3.26), most drastic differences were observed between the initial community and all incubations. In comparison to the initial community composition, differences among samples did not reveal apparent patterns of grouping by any factor.

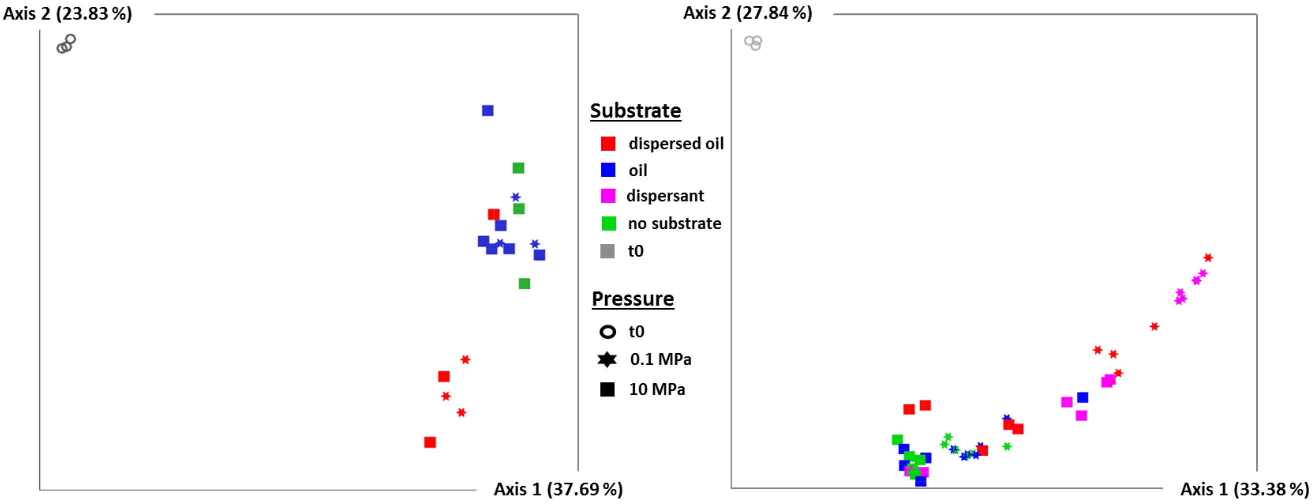


Fig. 3.26: PCoA plots (generated with Bray Curtis dissimilarity matrices) for the experiments Sept17B (left) and Feb18 (right). Color-coding for the substrate and form coding for incubation pressures, 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4°C, 168 h, n = 5 – 6, 200 rpm.

Taxonomy was assigned to the sequences of all runs with Greengenes database (99 %, Version 13_8), trained for the 16S rRNA sequence targeted in the amplicon sequencing. As discussed in chapter 3.2.3, the microbial communities changed drastically when incubated for 7 days. Starting compositions were more evenly distributed (Pielou's Eveness index, Fig. 3.25). Low abundant classes of bacteria were grouped as others (< 2 %; Sept17B: 34.1 ± 0.5 %, Feb18: 23.0 ± 3.2 %). Approximately half of the detected sequences in the sediment were assigned to the Proteobacteria (Sept17B: 47.4 ± 1.0 %, Feb18: 56.9 ± 3.6 %). In the starting community of Feb18 experiment Gammaproteobacteria (33.8 ± 3.3 %) and Deltaproteobacteria (11.9 ± 1.0 %) were already among the highest abundant classes. The same classes (Gamma- and Deltaproteobacteria) dominated the initial community of Sept17B experiment with 21.8 ± 0.4 % and 14.3 ± 0.2 % respectively (Suppl.Fig.2).

After incubation for seven days the community compositions changed drastically. In each experiment the drop in diversity (Fig. 3.25) was caused by an enormous average increase in Gammaproteobacteria's relative abundance to 80.7 ± 5.9 % and 86.7 ± 4.8 % (Sept17B and Feb18, respectively) of the whole community. While all the other initially high abundant classes decreased to low percentage values (Sept17B: 2.8 ± 0.6 % / 2.9 ± 1.0 %; Feb18: 2.9 ± 0.7 % / 2.0 ± 0.7 %, respectively Alpha- / Deltaproteobacteria).

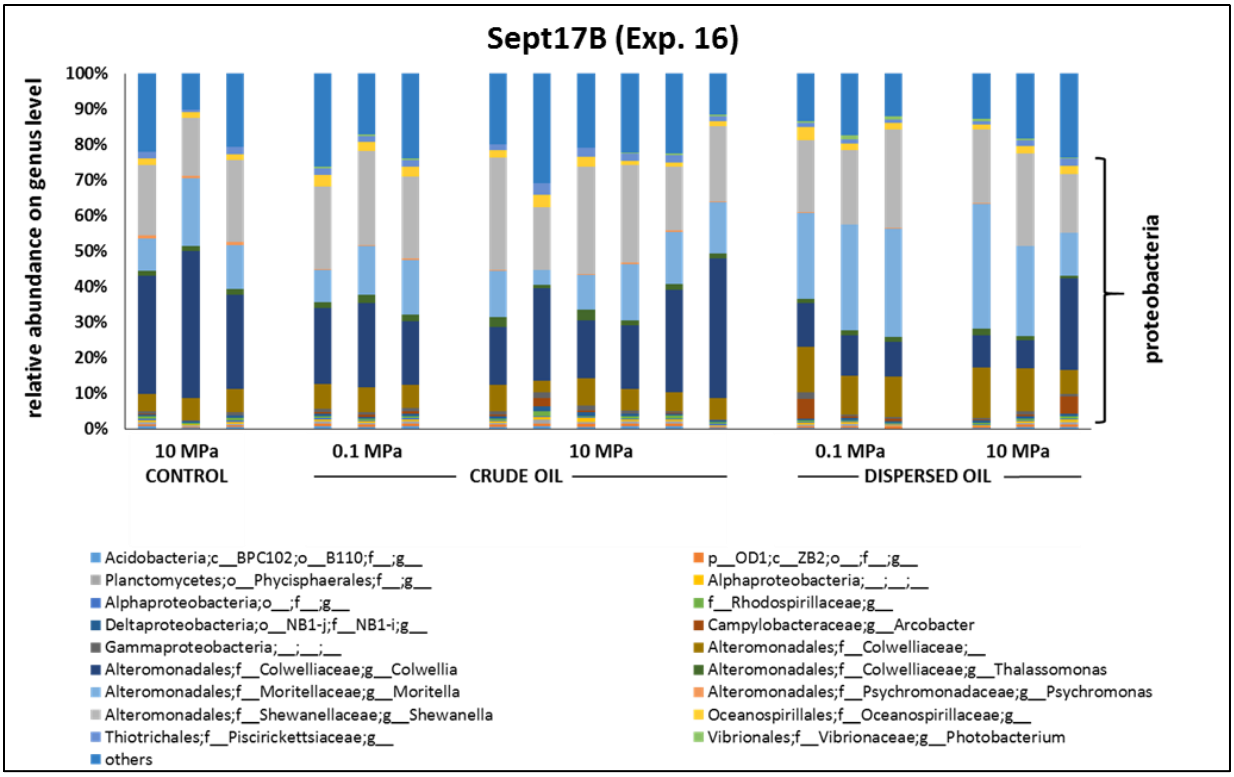


Fig. 3.27: Relative abundance on genus level (> 2 %) of all incubations (no t_0 samples shown) from experiment Sept17B. Taxonomy assigned with green genes (99 %) database release 13_8 (2013) trained for the primer pair applied in amplicon sequencing (341F – 785R). 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4°C, 168 h, n = 5 – 6, 200 rpm.

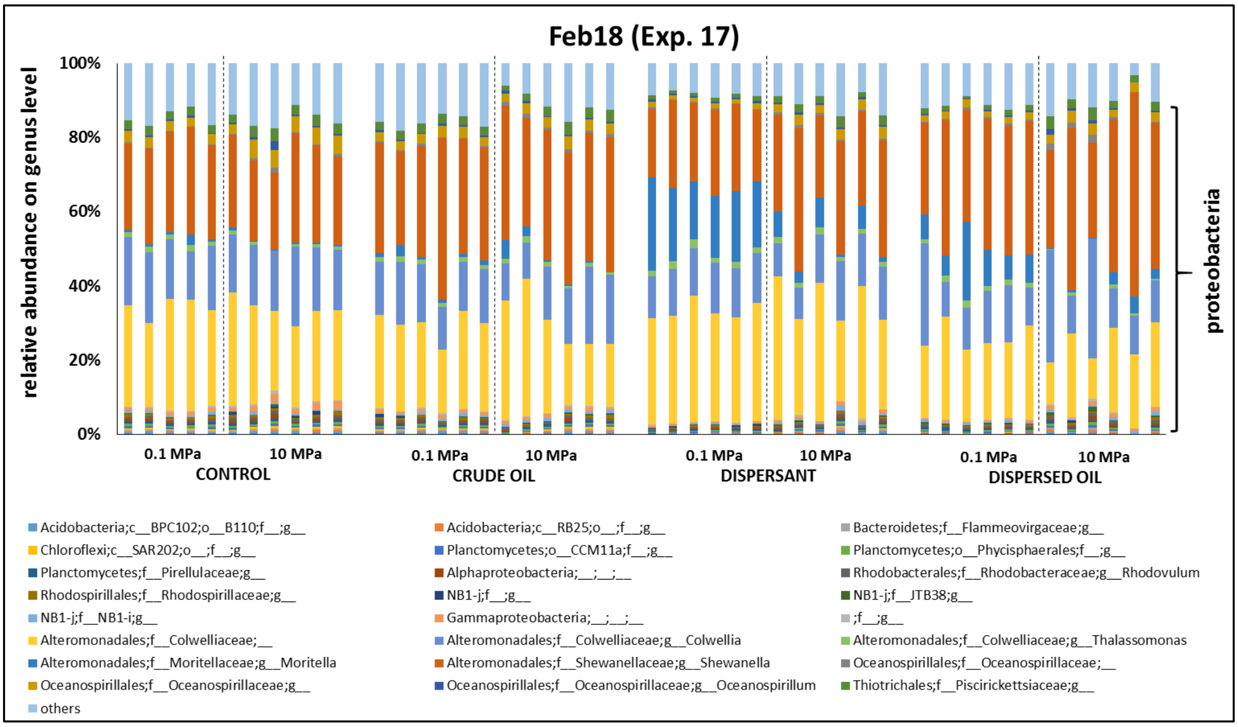


Fig. 3.28: Relative abundance on genus level (>2 %) of all incubations (no t_0 samples shown) from experiment Feb18. Taxonomy assigned with green genes (99 %) database release 13_8 (2013) trained for the primer pair applied in amplicon sequencing (341F – 785R). 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4°C, 168 h, n = 6, 200 rpm.

Regardless of the incubation conditions (pressure and substrate), all incubation were dominated by four genera (Fig. 3.27 (Sept17B) and Fig. 3.28 (Feb18)): an unidentified Colwelliaceae genus*, Colwellia, Moritella* and *Shewanella*. 69.1 ± 7.9% (Sept17B) or 75.0 ± 6.5% (Feb18) of all sequences were assigned to these four genera after incubation, starting from as little as 0.8 ± 0.1 % (Sept17B) and 1.8 ± 0.2 % (Feb18) in the initial community (t_0). Taking a closer look at these four genera, several variants (n = 11 – 18) were assigned to these taxa in experiment Feb18. Sequence variant analysis show detailed differences among the sequences assigned to the same representative sequence. Analysis of these variants with relative abundances higher than 0.01 showed no uniformly response to the conditions applied (Fig. 3.29). For example, *Moritella* variant v_3 and v_8 reacted strongly to the addition of dispersant at atmospheric pressure. *Colwellia* variant v_2 decreased in relative abundance with the addition of substrate at any pressure compared to the control. Unidentified Colwelliaceae variant v_1 were significantly more abundant when incubated with dispersant.

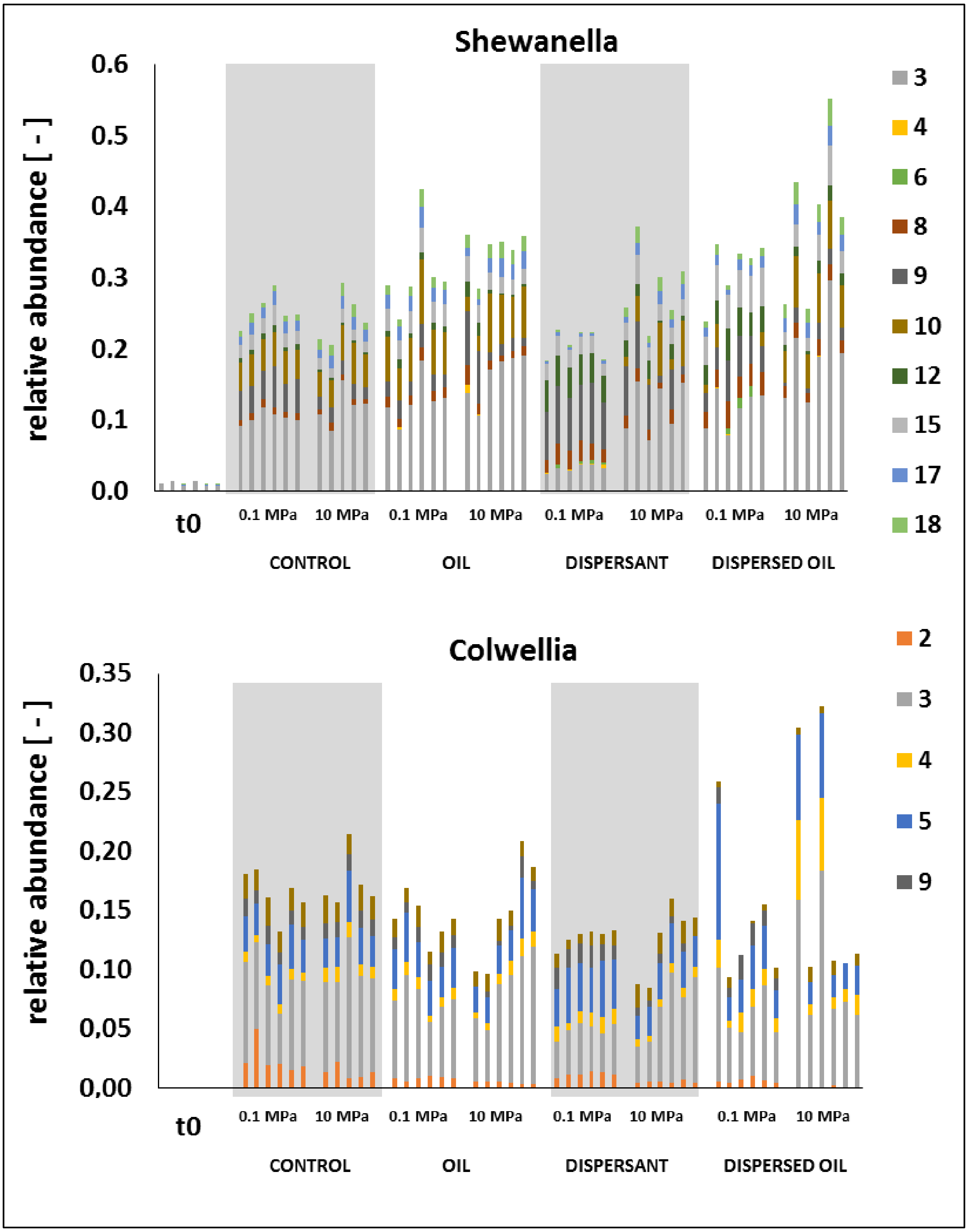


Fig. 3.29: Relative abundance of variants of the genera Shewanella *(n = 18) and* Colwellia *(n = 12). Feb18 / Exp. 17 (Tab. 2.5). Variants with max. relative abundance < 0.01 were omitted from the graphs. 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4 °C, 168 h, n = 5 – 6, 200 rpm. Color-coding for different sequence variants.*

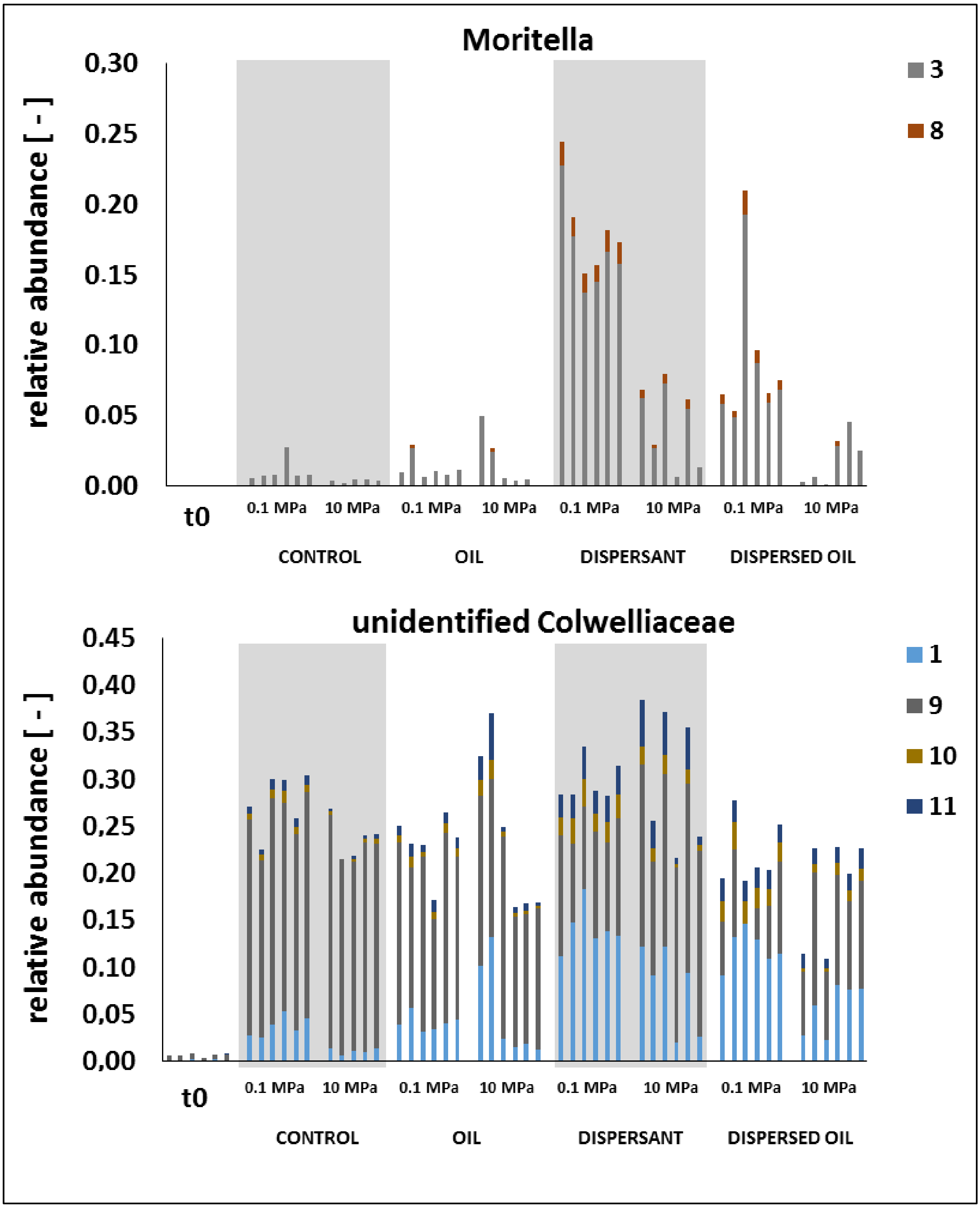


Fig. 3.29 continued: Relative abundance of variants of the genera, Moritella *(n = 13) and unidentified Colwelliaceae (n = 11). Feb18 / Exp. 17 (Tab. 2.5). Variants with max. relative abundance < 0.01 were omitted from the graphs. 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4 °C, 168 h, n = 5 – 6, 200 rpm. Color-coding for different sequence variants.*

Blasting those sequences with the NCBI database (Suppl.Tab.2) there where many high scoring hits with sequences also found in other studies on the sediments of the Gulf of Mexico (36 %) after the Deepwater Horizon explosion.

The drop in diversity reported for the DWH plume was also found in this study. Rivers et al. (2013) reported 95% of the 16S rRNA gene sequences in their samples belonged to only four families: Colwelliaceae, Oceanospirillaceae, and Piscirickettsiaceae, Methylococcaceae. In this study Colwelliaceae, Oceanospirillaceae and additionally Shewanellaceae made up 76.2 % of all 16S rRNA sequence reads (all t > 0 incubations, Feb18).

The community richness of taxa in the two identically designed experiments (Sept17B and Feb18) varied to a great extent. However, the bacterial species becoming dominant in the community were the very same with minor variations in the relative abundances. Sequence variant analysis revealed varying reactions to environmental factors within the dominant taxa, which are further discussed in Chapter 3.2.7.

3.2.5. Influence of Pressure on the Composition of the Bacterial Community

One of the essential questions in this thesis regarding deep sea top layer sediments was, whether parameter pressure influences the composition of the bacterial community. In general, the deep sea community is adapted to stable conditions with limited access to nutrients, carbon and energy sources.

The influence of pressure on the bacterial community was investigated with incubations of sediment without any substrate (control). There were two rationales for these controls: On the one hand, they were used as baseline for the incubations with complex substrate mixtures (Chapter 3.2.6). While on the other hand they allowed an evaluation of the isolated effect caused by the applied hydrostatic pressure on the microbial community.

Results of experiment Feb18 are more suitable for statistical analysis, as this experiment was conducted with sufficient repetitions (n = 6) of incubations with no substrate added at both pressures (0.1 / 10 MPa, Fig. 3.30).

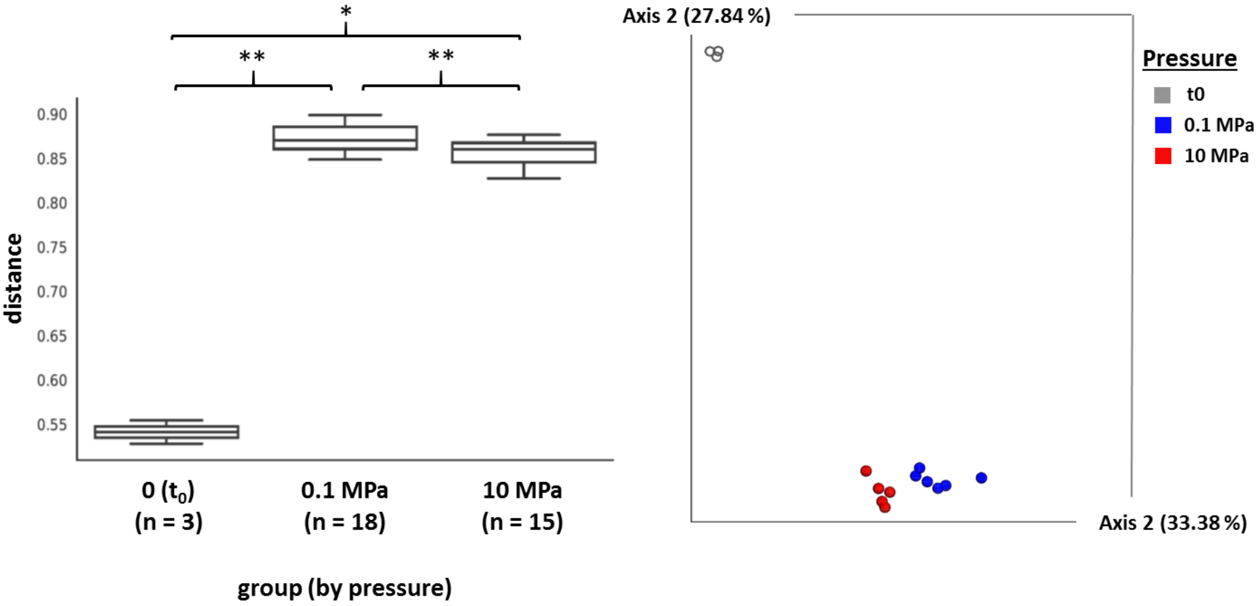


Fig. 3.30: left: Distance boxplot of the Feb18 (Exp. 17, Tab. 2.5) incubations at time zero and without any substrate for 7 days, at 4°C and 200 rpm with FSW. Group significance (Bray Curtis) was calculated with PERMANOVA and significance levels are indicated with ""($p < 0.05$) and "**" ($p < 0.01$). Right: PCoA plot (Bray Curtis dissimilarities) of t0 and control incubations (0.1 MPa = blue, 10 MPa = red).*

Feb18 incubations for seven days with filtered seawater (FSW) at both pressure resulted in significantly different community compositions amongst all groups (t_0, 0.1 MPa, 10 MPa). Comparing the incubations without any substrate but at different pressure also resulted in significant differences. The PCoA plot indicated a small, but distinct, separation by pressure (Fig. 3.30, right).

Comparing those bacterial communities which developed after incubations with the different substrates in the Feb18 experiment (oil, dispersant, dispersed oil) at 0.1 and 10 MPa, significant differences were found for the dispersant only and dispersed oil incubation ($p = 0.003$ and 0.004, respectively). While the crude oil incubation at different pressures did not result in significant differences ($p = 0.073$).

Hydrostatic pressure (0.1 and 10 MPa) had a significant influence on the bacterial communities' composition, without considering the effects of substrate. Considering the average depth of the oceans (4000 m, 40 MPa) the influence by constantly increasing pressure with depth is most likely increasing as well.

3.2.6. Influence of Substrate on the Composition of the Bacterial Community

The input of massive amounts of hydrocarbons, including crude oil and dispersant, led to drastic changes in the deep sea microbial community (Dubinsky et al., 2013, Rivers et al., 2013) during the DWH oil spill.

In the two main experiments, Sept17B and Feb18, crude oil (1 % v/v) was added to the incubations of sediments with and without the addition of dispersant (1:100 DOR). Incubations without any substrate added were run in parallel to evaluate the influence of substrates on the bacterial communities by comparison.

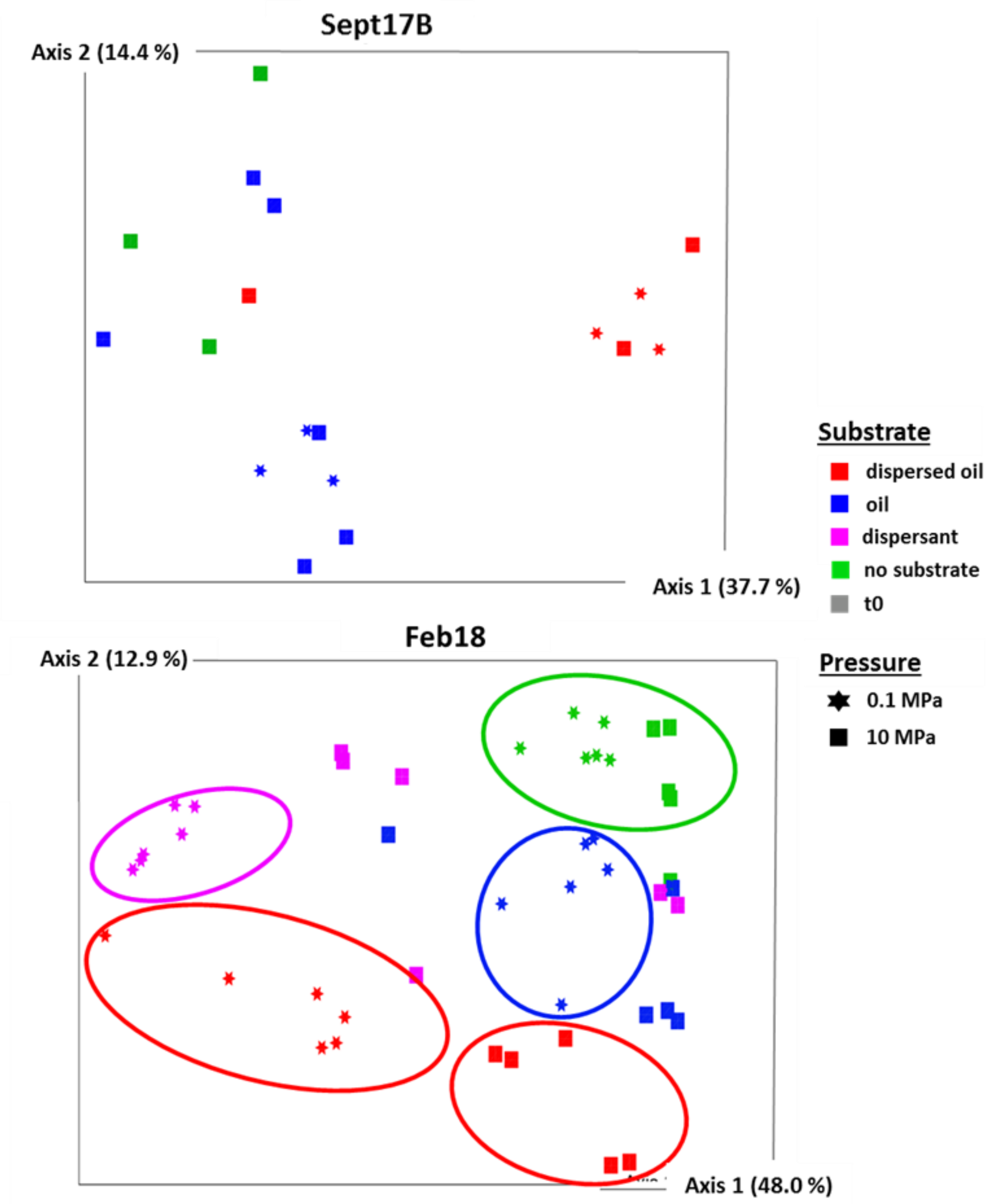
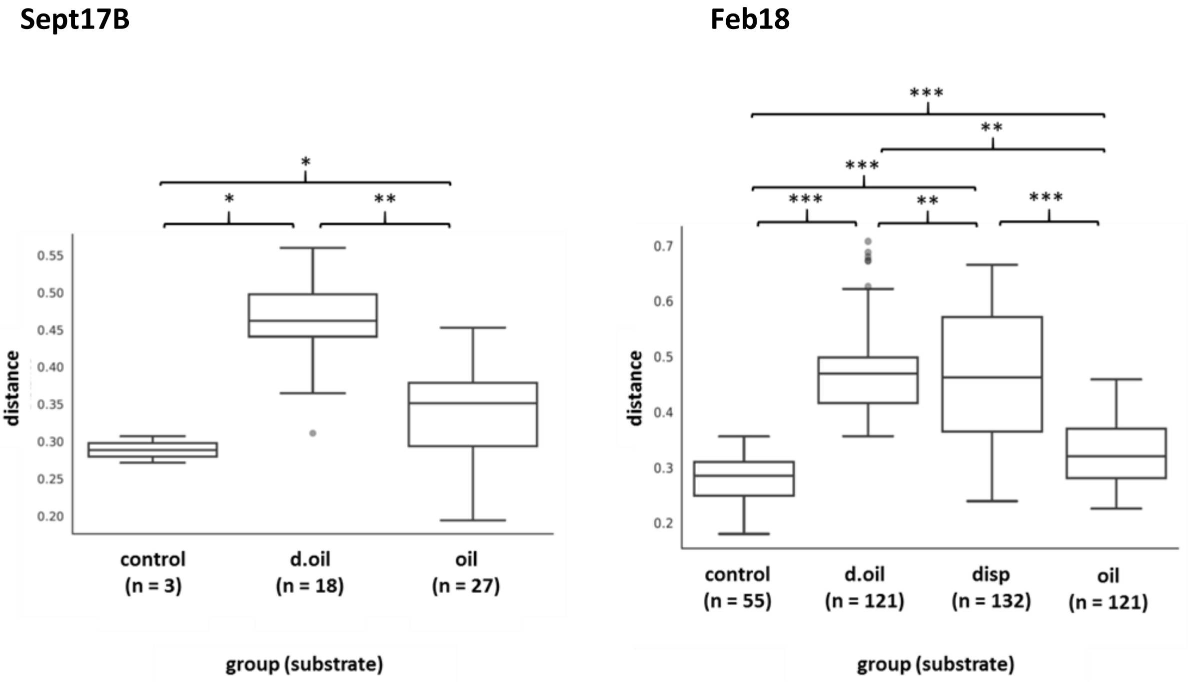


Fig. 3.31: PCoA plots (generated with Bray-Curtis dissimilarity matrices) for the experiments Sept17B (above) and Feb18 (down), without the t_0 samples. Color-coding for the different substrate and form-coding for different incubation pressures, incubation for 168 h, 4 °C, 200 rpm with FSW.

Besides the obvious differences between the initial community compositions (t_0) and the composition of the community of any incubation ($t > 0$ h), the differences within the group of incubations were more complex. Regardless of the pressure, differences were detectable between the substrates added in each experiment (Sept17B and Feb18). For a new calculation of PCoA (Sept17B and Feb18) the t_0 samples were omitted. Sept17B experiment samples were poorly grouping with the substrates (Fig. 3.31 above). Feb18 samples were mostly clustering by the substrates added, except for scattered distribution of the crude oil only and the dispersant only samples at 10 MPa (Fig. 3.31 below).

To test the significance of the differences between the incubations (PERMANOVA, Bray-Curtis dissimilarities, 999 permutations) only the substrate as variable was taken into consideration. In other words, all pressure incubations were pooled by the substrate added. Results revealed significant differences among all substrate incubations. The Feb18 experiment was conducted with a minimum of five samples per substrate and pressure, which made a more detailed analysis possible. Testing differences (Fig. 3.32 right, PERMANOVA, Bray-Curtis dissimilarities, 999 permutations) pairwise, without taking pressure into consideration, most significant differences ($p \leq 0.001$) were found between the control samples and all other substrate samples, as well as for samples with dispersant and with oil alone ($p = 0.001$).


Sept17B

Feb18

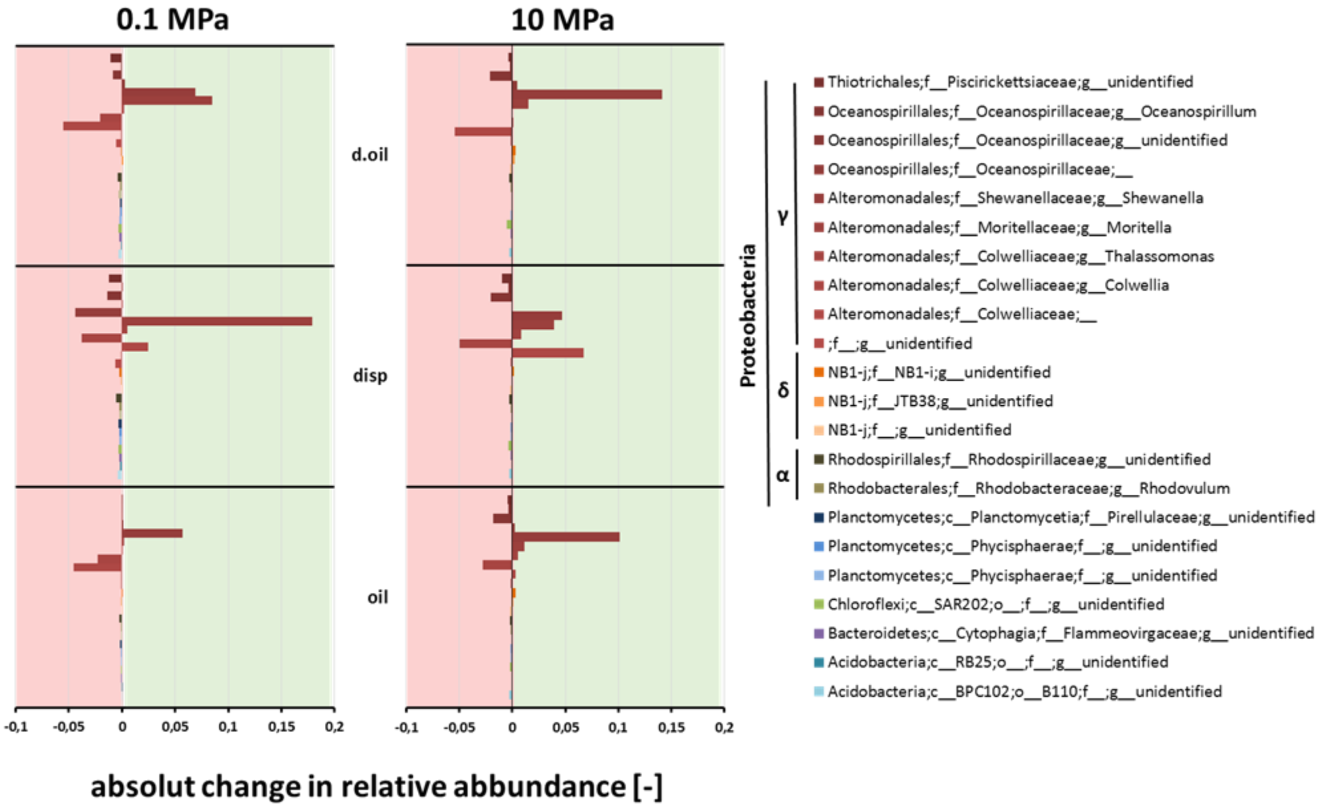
Pairwise permanova results (Sept17B)

Group 1	Group 2	Sample size	Permut.	pseudo-F	p-value	significance
d.oil	none	9	999	4.97	0.014	*
	oil	15	999	5.18	0.002	**
none	oil	12	999	1.75	0.049	*

Pairwise permanova results (Feb18)

Group 1	Group 2	Sample size	Permut.	pseudo-F	p-value	significance
d.oil	disp	23	999	3.78	0.016	**
	none	22	999	10.86	0.001	***
	oil	22	999	5.46	0.003	**
disp	none	23	999	12.66	0.001	***
	oil	23	999	9.64	0.001	***
none	oil	22	999	4.03	0.001	***

Fig. 3.32: Distance boxplots of the substrate classes of experiment Sept17B and Feb18 and pairwise PERMANOVA results (Bray Curtis dissimilarity matrices). 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4 °C, 168 h, n = 5 – 6, 200 rpm. cotrol – no substrate added, disp. – dispersant, oil – crude oil, d.oil – dispersed oil.



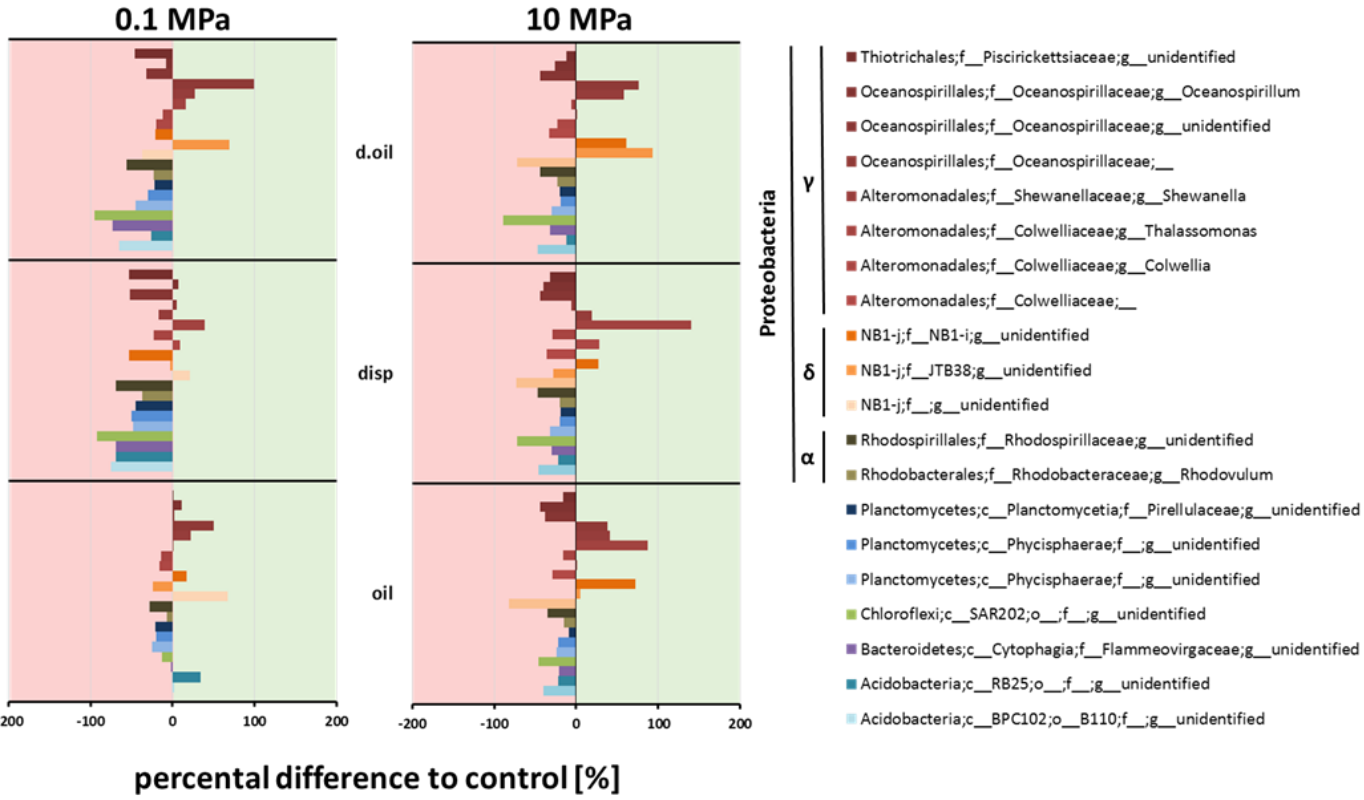


Fig. 3.33: Average absolute (above) and percentage (below) change of the genera's relative abundance of substrate incubations (crude oil (oil), dispersant (disp), dispersed oil (d.oil)) in comparison to the no substrate control (control 0.1/10MPa = 0 / 100 %) of the most abundant families (> 2 % of all sequences) at each pressure from Feb18 experiment. Due to high percental increase (0.1 MPa: d.oil = 791.9 %, disp = 1676.7 %, oil = 18.8 %; 10 MPa: d.oil = 368.8 %, disp = 975.5 %, oil = 278.2 %) Moritella *is omitted from the graph below.*

The taxa promoted by substrate addition were evaluated by comparing samples with the different substrates to the incubation without any substrate added and run for the same time (7 days, biological control, Fig. 3.33). Changes towards more relative abundance (> 0 % of control) were found in the Delta- and Gammaproteobacteria, and for one of the Acidobacteria with crude oil at atmospheric pressure. Significant changes in the relative abundance of genera were more numerous in the atmospheric pressure incubations. While significant increases at atmospheric pressure were exclusively detected for *Moritella* (892 % and 1777 %; d.oil and disp incubation), *Shewanella* (122 %; crude oil incubation) and the unidentified *Oceanospirillaceae* genus (113 %; d.oil incubation). The *Oceanospirillaceae* genus' relative abundance was at a low level at any pressure and incubation (maximum: 0.029 (oil 10 MPa)). Even though changes in elevated pressure incubations were high (on average: 1076 %) for *Moritella*, these were not found to differ significantly (Wilcoxon Rank Sum Test, Suppl.Tab.3), probably due to their low relative abundance at elevated pressure (maximum: 0.044 ± 0.028 (disp)).

The most pronounced changes at any pressure were detected in the taxa *Moritella,* which increased from an average relative abundance of 0.011 ± 0.008 (control) to 0.190 ± 0.031 (disp) and 0.095 ± 0.054 (d.oil), at atmospheric pressure. At elevated pressure, *Moritella* changed from even less in the control (0.004 ± 0.001) to 0.044 ± 0.028 (disp) and 0.019 ± 0.016 (d.oil) in relative abundance. When incubated with crude oil alone, *Moritella* did not increase in a similar manner (0.013 ± 0.008 (0.1 MPa) / 0.015 ± 0.018 (10 MPa)).

Shewanella were among the most abundant genera in the control treatment (0.260 ± 0.018 (0.1 MPa) / 0.244 ± 0.031 (10 MPa)), but did increase in all treatments with substrate added, except for the incubation with dispersant at atmospheric pressure. Relative abundances of *Shewanella* were at a maximum level, whenever crude oil was present in the incubations (0.1 MPa: oil = 0.316 ± 0.057, d.oil = 0.329 ± 0.042; 10 MPa: oil = 0.345 ± 0.025, d.oil = 0.385 ± 0.102). Dispersant, as sole carbon source added, did not lead to an increase in the relative abundance of *Shewanella* (0.1 MPa = 0.216 ± 0.020, 10 MPa = 0.291 ± 0.053).

Except for the incubation with dispersant alone (0.1 MPa = 0.129 ± 0.007, 10 MPa = 0.125 ± 0.029), relative abundance of *Colwellia* did not change significantly when incubated with substrate (both pressures range: 0.143 – 0.176), compared to the control incubation (0.1 MPa = 0.167 ± 0.019, 10 MPa = 0.175 ± 0.020). In contrast, an unidentified Colwelliaceae genus showed slightly increased relative abundances compared to the control (0.1 MPa = 0.277 ±

0.028, 10 MPa = 0.238 ± 0.019), exclusively in the incubation with dispersant alone (0.1 MPa = 0.302 ± 0.021, 10 MPa = 0.305 ± 0.068). The unidentified Colwelliaceae genus decreased in all other incubations at both pressures (range: 0.184 – 0.241).

In general, a decrease in relative abundance of genera was much more often observed (79 out of 120; Suppl.Tab.4) with any substrate added. Especially the unidentified SAR202 genus (Chloroflexi) decreased significantly with any dispersant added (either alone or in dispersed oil) to lowest values recorded (4.7 % of control, d.oil at 0.1MPa). In a similar manner, many families (e.g. Flammeovirgaceae, Acidobacteria, Piscirickettsiaceae) did decrease significantly with dispersant or dispersed oil added, while the in/decreases with oil only were not significant. This pattern was again more pronounced at atmospheric pressure.

Some families behaved in an ambiguous way. For the Colwelliaceae, *Colwellia* mostly decreased significantly with dispersant only added at both pressures, while *Thalassomonas* increased in relative abundance, except for one incubation. Also for the Oceanospirillaceae there was no uniform reaction to the addition of substrate among (mostly unidentified) genera.

Several known hydrocarbon degrading genera (Yakimov et al., 2007, Head et al., 2006) were found in this study: *Oceanospirillum* (395 sequences; max. rel. abund: 0.024), *Arcobacter* (158 sequences; max. rel. abund: 0.009), *Neptunomonas* (36 sequences, max. rel. abund: 0.002) were found in this study, along with 3777 sequences assigned to the Oceanospirillales (max. rel. abund: 0.051). This order is assumed to have played an important role in crude oil degradation after the DWH oil spill by responding quickly to aliphatic hydrocarbon input (Mason et al., 2012, Redmond and Valentine, 2012).

All substrates had a significant influence on the bacterial community. The major effect observed at both pressures was a decrease in relative abundance (65.8 % of all genera relative abundance). Most of those genera decreasing in abundance did decrease more extensively when dispersant was present (disp / d.oil, Suppl.Tab.4).

A significant increase in relative abundance was only observed for the genera *Moritella* and *Shewanella* and one unidentified Oceanospirillaceae genus. However, their response to the substrate addition differed: *Moritella* did only increase substantially in relative abundance, when dispersant was added. While *Shewanella* was already highly abundant in the control treatments, but increased in relative abundance, especially when oil was present.

***Shewanella* spp. have been reported to degrade crude oil and it components (Deppe et al., 2005, Gerdes et al., 2005) and they were active after the DWH incident (Joye et al., 2016, Gutierrez, 2017).**

On the other hand, *Moritella* spp. are known to inhabit the deep biosphere (Sogin et al., 2006, Lauro and Bartlett, 2007), but so far they are not considered a crude oil or dispersant degrading taxa. *Moritella* was found in small abundances in isolation studies on oil slicks of the DWH (Gutierrez et al., 2016) and have been mentioned in studies on plastic degrading deep sea bacteria (Sekiguchi et al., 2013).

3.2.7. Influence of Multiple Factors (Pressure, Oil, and Dispersant) on the Microbial Community

Separate investigation on the parameters pressure and substrate revealed significant influence by these factors (chapters 3.2.5 and 3.2.6). To integrate pressure and substrate simultaneously in the analysis, all pressure and substrate groups were tested for differently abundant taxa (ANCOM analysis, Mandal et al., 2015) in the Feb18 experiment. As changes occurred just by the incubation setup, the initial samples (t_0) were neglected and the samples were compared to the control samples. In brief, ANCOM tests for significant different abundant sequence variants and gave a W-value and a value above which the H_0 hypothesis (= equally abundant along groups) was rejected (Tab. 3.2).

All samples were grouped by pressure and substrate (e.g. d.oil 10 MPa, n = 6) for this analysis. The differently abundant taxa found were all among the Alpha- and Gammaproteobacteria, as well as the Bacteroidetes. Most known oil degrading species belong to these phyla (Prince et al., 2018).

Reject null hypothesis	W-value	Taxon (gg_99 trained classifier)	Confidence	Known variants (Fig. 3.29)
True	3082	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Shewanellaceae; g__Shewanella; s__	0.933	Shewanella Variant 10
True	3080	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Colwelliaceae; g__Colwellia	0.906	Colwellia Variant 9
True	3080	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Moritellaceae; g__Moritella; s__	1.000	Moritella Variant 8
True	3074	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Colwelliaceae; g__Colwellia	0.898	Colwellia Variant 2
True	3064	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__; g__; s__	0.805	
True	3052	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Shewanellaceae; g__Shewanella; s__	0.815	Shewanella Variant 1
True	3048	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria	1.000	
True	3032	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kordiimonadales; f__Kordiimonadaceae; g__; s__	0.998	
True	3006	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Colwelliaceae	1.000	
True	3005	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales; f__Oleiphilaceae; g__; s__	0.999	
True	3001	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Moritellaceae; g__Moritella; s__	1.000	Moritella Variant 9
True	2998	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Moritellaceae; g__Moritella; s__	1.000	Moritella Variant 3
True	2993	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Colwelliaceae; g__Thalassomonas; s__	0.818	
True	2929	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Cryomorphaceae; g__; s__	0.778	
True	2904	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales; f__Halomonadaceae; g__Cobetia; s__	0.989	Cobetia Variant 2
True	2870	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Shewanellaceae; g__Shewanella; s__	0.823	Shewanella Variant 12
True	2845	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Colwelliaceae; g__Thalassomonas; s__	0.711	
True	2823	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kordiimonadales; f__; g__; s__	0.826	
True	2819	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__; s__	0.945	

Tab. 3.2: ANCOM result of Feb18 experiment without t0 samples. All significant different abundant variants are shown. Variants already named are listed as “known variants” from Fig. 3.29.

The most differentially abundant variants were assigned to the order of Alteromondales. Many of the variants found by ANCOM analysis have already been described when looking for the most abundant species (Fig. 3.29).

To further investigate these variants their average relative abundance was calculated and grouped by taxonomic relation (Fig. 3.34). One variant of *Moritella* (*Moritella* v_3) and the two variants assigned to the order of Kordiimonadales were responding to the addition of dispersant. While all *Moritella* variants increased in relative abundance, all Kordiimonadales were reduced (or no sequences detected) when dispersant was added to the incubation. In the same way, *Moritella* v_3 seemed to be less dominant in the community, when pressure was applied. On the other hand, Kordiimonadales were only abundant with dispersant present, when pressure was applied.

The two variants assigned to Flavobacteriales families showed an opposing behavior when substrate was added, while the Cryomorphaceae variant was not detected with any substrate added, the Flavobacteriaceae variant mainly occurred when substrate was added. *Cobetia* v_2 and Oleiphilaceae behaved similarly by increasing with substrate, but only at atmospheric pressure. Less obvious responses were observed with *Shewanella* and *Colwellia* variants. While *Shewanella* variant v_10 seemed to decrease with dispersant added to the incubations, *Shewanella* variant v_12 seemed to increase. *Shewanella* variant v_1 was only present, when dispersant was added at atmospheric pressure. *Colwellia* variants did not show a clear trend on any parameter combination. Only *Colwellia* variant v_3 and v_4 were increasing in relative abundance when dispersant, oil and pressure are present, while all others were decreasing (Fig. 3.29), especially *Colwellia* v_2.

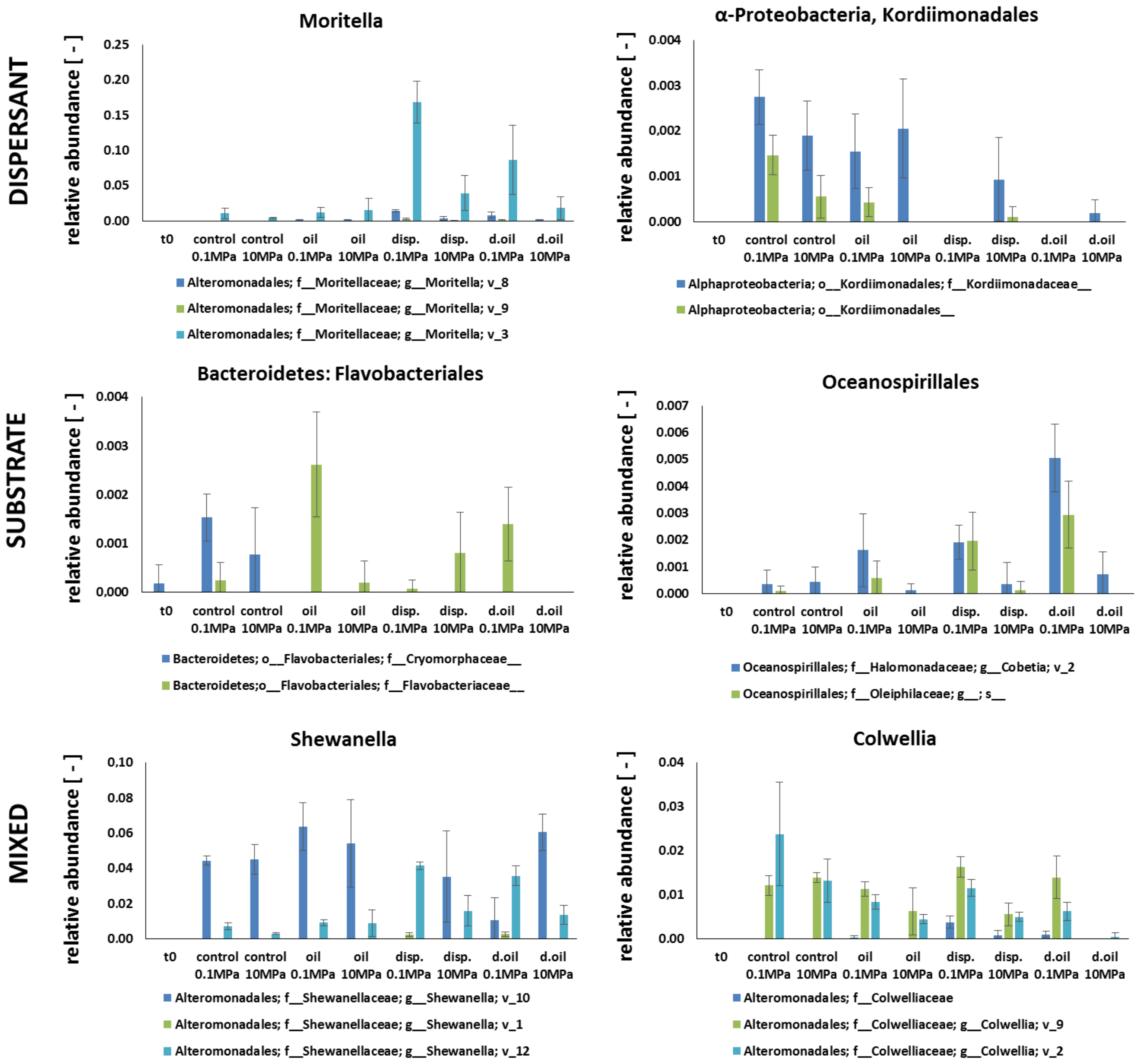


Fig. 3.34: Average relative abundance of variants being significant differently abundant (ANCOM) grouped by relation. Lines are grouped by responses (dispersant, substrate, mixed). 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4°C, 168 h, n = 5 – 6, 200 rpm.

Interestingly, just few of the significantly different abundant variants (except *Shewanella* v_3, *Colwellia* v_3 and v_4) were observed to have a peak in relative abundance in the dispersed oil incubation (d.oil) at 10 MPa. In some cases, a single variant reached high relative abundances (*Shewanella* v_3; d.oil, 10 MPa: 0.192 ± 0.057).

This stresses the fact, that the presence of a certain genus, is no proof of its role in the degradation of crude oil, as the ability to metabolize crude oil is obviously differing by

variants. Variants are different organisms on a sub-species / -OTU level, and can be referred to as ecotypes (oligotypes).

As argued before (Kleindienst et al., 2016), the microbial communities responses to the DWH spill might promote single ecotypes, which are specialized in degradation of crude oil components under specific environmental conditions.

So far the analysis of all applied factors was done separately (pressure, substrate). As those factors are acting simultaneously during the incubations, the weight of each environmental factor (pressure, oil, dispersant) was evaluated in an Ordinary Least Square (OLS) model (Tab. 3.3, Bray Curtis dissimilarities, Feb18). Calculation of the model followed the “Differential abundance analysis with gneiss” instructions for QIIME2.

No. Observations	470.000	
Model:	**OLS**	
R^2:	**0.16**	
factor	**mse**	**R2diff**
intercept	101.87	0.69
pressure	59.13	0.05
dispersant	61.75	0.09
oil	57.70	0.03
Cross-validation	**model_mse**	**pred_mse**
fold_0	417.74	74.85
fold_1	427.06	62.57
fold_2	428.75	59.83
fold_3	427.20	62.90
fold_4	419.92	70.37
fold_5	428.87	59.64
fold_6	438.82	51.09
fold_7	427.70	61.69
fold_8	428.64	59.94
fold_9	436.14	55.99

Tab. 3.3: Gneiss-OLS model output on Feb18 Bray Curtis dissimilarities (level 6 – genus). R^2*: regression coefficient for the whole model.* R^2*diff: regression coefficient for the single factor (covariant) quantifying explanatory influence. Cross-validation comparing within model mean square errors (model_mse) and prediction accuracy (pred_mse).*

Little of the variances can be explained by this OLS model, as the regression coefficient (R^2) is quite low (0.16), but within the range of ecological studies. Evaluating the influence of the single factors on the variances (R^2diff), dispersant is explaining the most of the variation with 9 %, while pressure accounts for 5 % and the addition of crude oil for 3 %. The cross validation gives insight into over-fitting of this model. As the prediction accuracy is lower than the within model error with every repetition, there is no indication for an over-fitting with the three factors.

The low regression coefficient (R^2 = 0.16) of the OLS model showed that the three investigated factors (pressure, oil and dispersant) are insufficient to model a complex system like the deep sea microbial communities changes. Many important environmental factors, like biotic interactions or important biogeochemical parameters, have not been included in the model. What can be deduced from this model is a ranking of the influence of investigated factors. This revealed dispersant as the most impacting factor on the bacterial communities composition.

3.2.8. Error Analysis

Gulf of Mexico deep sea sediments from two cruises (2016 and 2017) were used in this study. The sediment was depressurized and shipped and stored at near *in situ* temperatures (4 °C) for different time periods. Together with the thoroughly mixed subsample of the sediment samples, might have led to an artificial inhomogeneous community composition, which might explain variations in the initial community compositions (Fig. 3.15). Furthermore, the problems resulting from handling viscous crude oil and small amount of dispersant might have led to differences in the substrate added.

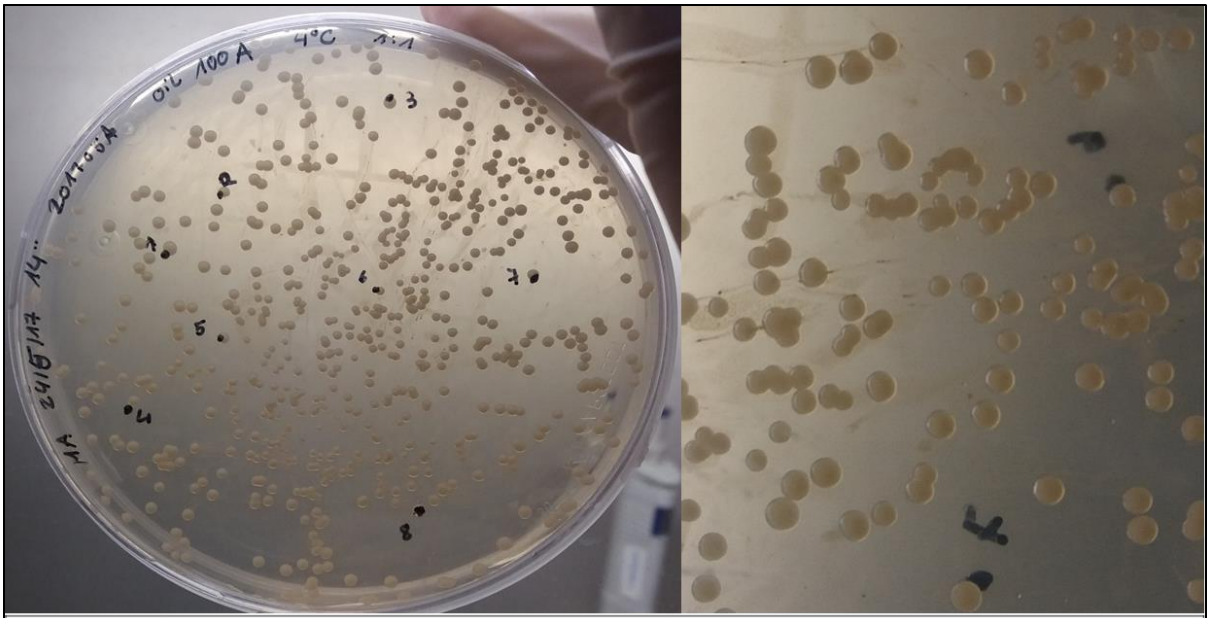
The established method of sequencing highly conserved 16S rRNA regions for identification of prokaryotes heavily depends on the applied primer set and is limited by amplification of short read length, sequencing errors, the comparability of different regions used and the broad variety of methods to obtain OTUs (Poretsky et al., 2014). Furthermore, the analysis is a strict relative method, which depends on a proper amount of successful reads. This amount varied to some extend in this study ($3.92*10^3$ and $2.11*10^5$ reads / sample). DNA was extracted from a subset of the incubation medium before thorough mixing, but might just represent an inhomogeneous blended part of the incubation.

3.3. Isolation of a Representative Hydrocarbon Degrading Bacterium From an Impacted Sediment Community (*Cobetia* sp. C6)

Isolation procedures were applied following the community experiments with the northern GoM surface sediment mixture at elevated pressure. Amplicon (16S rRNA) sequencing is able to reveal relative abundances for the whole community, but no (reliable) information on absolute cell numbers or metabolic activities were acquired. Therefore, isolations were performed to gather detailed information on representative hydrocarbon degrading single strains from these incubations.

3.3.1. Isolation Procedure Leading to a Pure *Cobetia* Culture

Experiment May17A was conducted as described before (Fig. 3.35). After incubation with crude oil (and methane) at 10 MPa (replicate 1 of 2), 100 µL of the incubation mixture were spread plated on marine agar (MA) and incubated at 4 °C for a week.
The grown colonies were picked and the partial 16S rRNA was analyzed (Fig. 3.35). 16S rRNA sequences of nearly all picked colonies (C2 – C7) were identical (341F – 907R primer: 100 %) to the isolate *Cobetia* sp. 7523M (NCBI accession: KU666953). The two other colonies (C1 / C8) also shared a high identity with *Cobetia* sp. 7523M (99 %). Hence, these isolates belong most probably to the same enriched species.



Colony (plate)	Species	Accession number	Query cover	Year	Author(s)
1	Halomonas sp. strain PS081_2009	KY388991	100%	2017	Raverty,S.A., et al
2	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
3	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
4	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
5	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
6	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
7	Cobetia sp. 7523M	KU666953	100%	2016	Laport,M.S.
8	Cobetia sp. strain FED_03A	KY020309	100%	2016	Izzo,S.A.

Fig. 3.35: Pictures and 16S rRNA (314F / 907R) identification of the eight colonies obtained from spread plating of experiment May17A (Exp. 13, Tab. 2.5) with oil at 10 MPa. Identification with reference with referring accession number from the NCBI database (https://www.ncbi.nlm.nih.gov/).

The genus *Cobetia* was detected in the community analysis of the May17A experiment, but only up to 0.08 % of the whole community. In the t_0 sample and the incubations without substrate added, no sequences assigned to *Cobetia* were detected, while a relatively large proportion (0.079 %) was found in the oil incubations at atmospheric pressure (Fig. 3.36). In one of the two samples with crude oil at deep sea pressure (10 MPa) 0.011 % (11 of 94552) of the sequences were assigned to the genus *Cobetia*. From this sample the isolation was initiated by spread plating.

In all other sediment experiments (Exp. 10 – 12, 14 – 17; Tab. 2.5) the genus *Cobetia* was found to increase in relative abundance from initial incubation (t_0). Starting conditions with nearly no detectable abundance (average 0.01 ± 0.02 %; max. 0.07 %) of the community increased in all incubations up to an average of 0.17 ± 0.31 % (max.: 1.74 %).

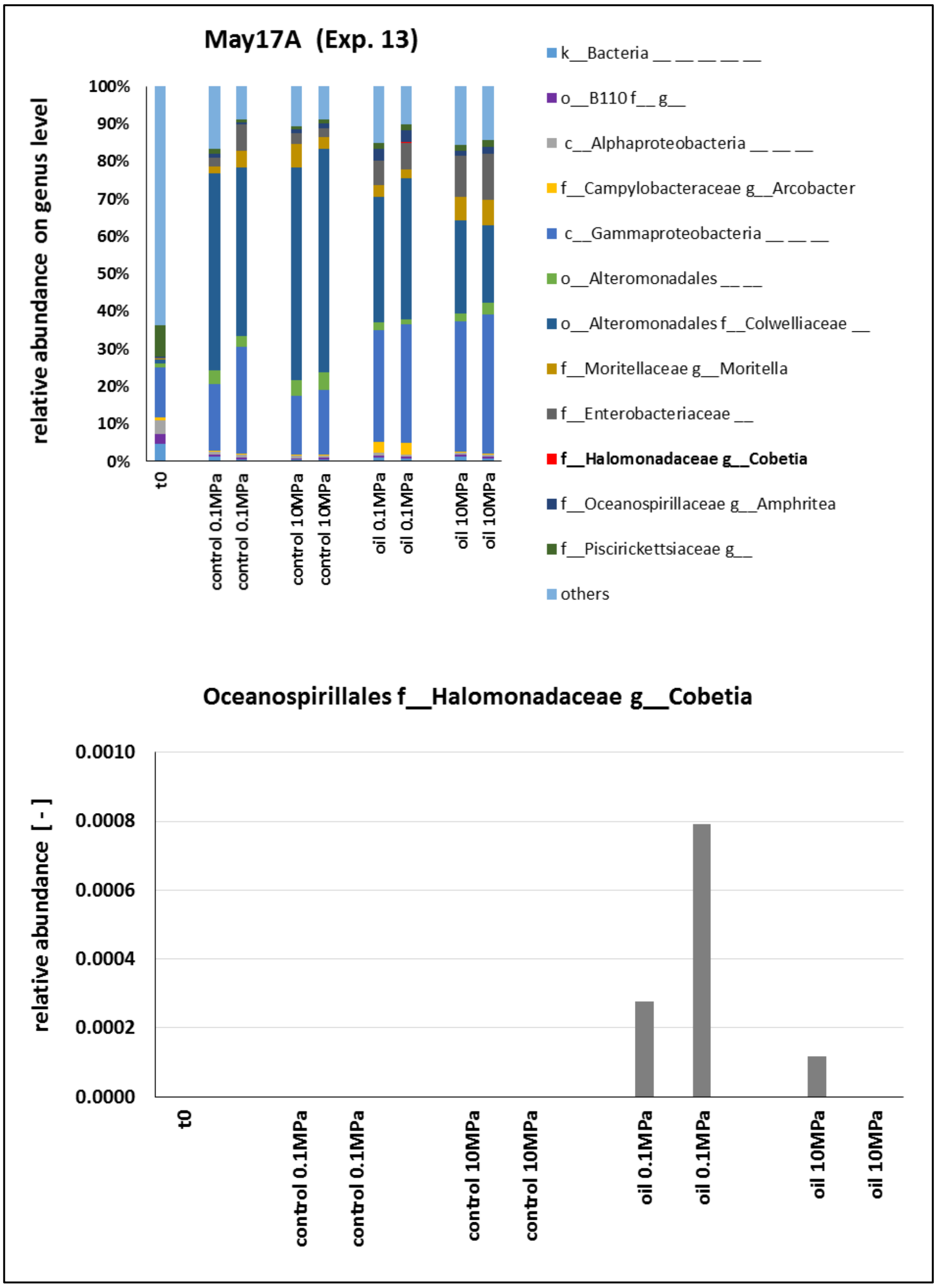


Fig. 3.36: Left: relative abundance of May17A experiment incubations on genus level. Right: relative abundances for only genus Cobetia in all experiments. 20 mL ASW, 200 µL crude oil (1 % v/v), 4 °C, 168 h, n = 3, 200 rpm.

Only this one single genus with a very small abundance (0.011 %) of the whole community was favored by the condition provided by the MA agar plates. Differences in pressure and the specific nutrient concentrations provided by the medium have led to the selection and isolation of the *Cobetia* sp. strains. The inability to cultivate the great majority of organisms

by isolation methods is known as the "Great Plate Count Anomaly" (Amann et al., 1995; Brock, 1989, Tab. 3.4).

The attempt to gain absolute abundances of sediments (chapter 3.2.2) used the same agar, marine agar (MA). There is a high chance, that those colonies, which grew in the sediment experiments, were exclusively *Cobetia* organisms.

Considering the isolation conditions, the isolated *Cobetia* strains are most probably able to thrive at atmospheric pressure and under the presence of complex substrates.

Habitat	Culturability (%, CFU)
Seawater	0.001-0.1
Freshwater	0.25
Sediments	0.25
Soil	0.3
Acivated sludge	1-15

Tab. 3.4: Prokaryotic culturability (as % CFU) of different habitats. Adapted from Amann et. al (1995).

3.3.2. Growth and Nutrient Requirements of Isolated *Cobetia* Strains

The *Cobetia* isolates were obtained from the May17A incubation (Exp. 13, Tab. 2.5) with crude oil added. For this reason, a toxic effect of crude oil towards the strains is unlikely. To show that the strains were able to metabolize crude oil, all the colonies were incubated in parallel with crude oil as carbon source at 4 °C and at atmospheric pressure in ASW.

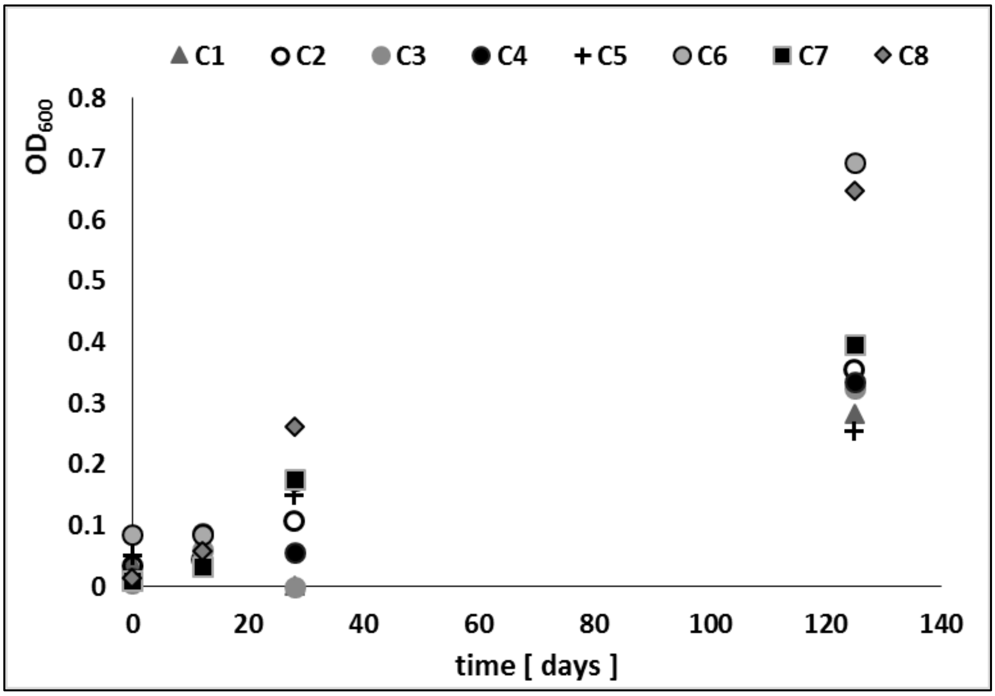


Fig. 3.37: Optical density of the eight Cobetia *sp. isolates C1 – C8 in 20 mL ASW with 200 µL crude oil at 4 °C and 200 rpm for 125 days.*

Resulting growth of all the isolates was very low with all optical density values (OD_{600}) below 0.100 after 12 days. Due to this sustaining low overall growth (max $OD_{600\ 28d}$ = 0.263 (C8)) the comparably fastest and best growing strains (C6 / C8) were picked for further experiments (Fig. 3.37).

The ASW medium used in the isolation and some sediment experiments contained nutrient concentrations resembling the oligotrophic environment of the deep sea (PO_4^{3-}/ NO_3^-: 2 µM / 20 µM). An investigation on the nutrient requirements of *Cobetia* sp. C6 and C8 was conducted by testing three different concentrations of nitrogen (NO_3^- / NH_4^+) and phosphorus (PO_4^{3-}) sources (Fig. 3.38). Both isolates were able to grow with any medium within 5 days of incubation. Growth rates were significantly higher with the medium providing the highest nutrient supply (μ_{MM2}: C6 / C8 = 0.039 / 0.025 h^{-1}) compared to the media without (no P/N source) and ASW nutrient supply (range $\mu_{no\ P/N\ source}$ and μ_{ASW}: 0.004 – 0.015 h^{-1}). These results led to the choice of the medium in subsequent experiments, which supported growth to the highest extent (MM2).

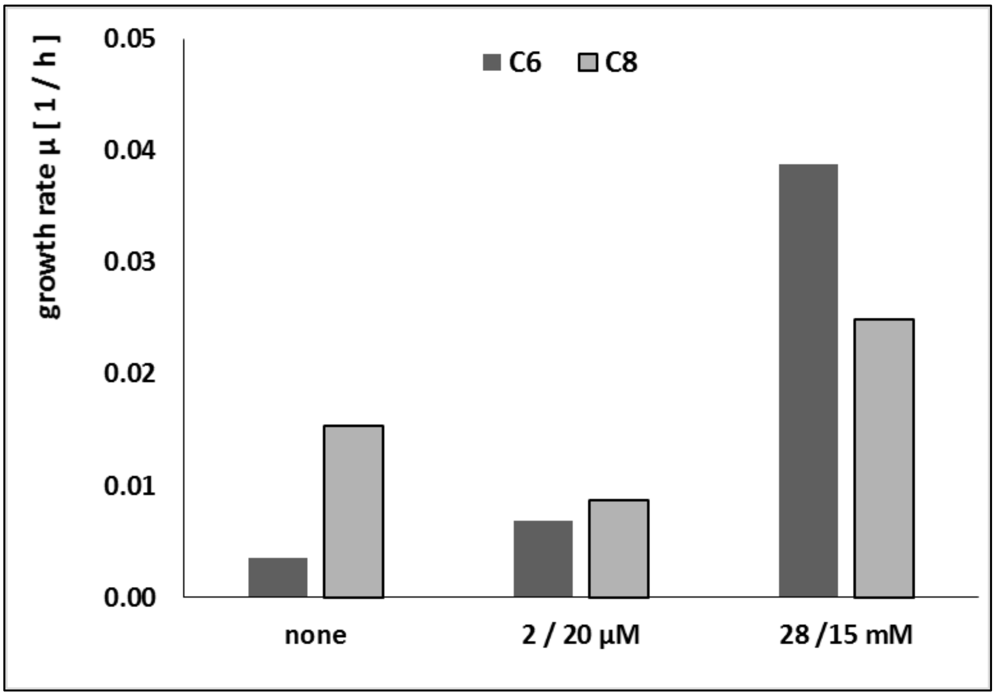


Fig. 3.38: Incubation of Cobetia C6 / C8 with 20 mL medium with different nutrient concentrations ([PO_4^{3-}] / [NO_3^-]: - / - ; 2 / 20 µM ; 28 / 15 mM), 1 % v/v crude oil (200 µL) at RT for 117 h.

Investigation of both isolates with the addition of crude oil resulted in small growth rates of *Cobetia* sp. C6 and C8 (C6/C8: 0.010 h^{-1} / 0.008 h^{-1} , Fig. 3.38). Even smaller growth rates were detected when dispersed oil was added (C6/C8: 0.002 h^{-1} / 0.004 h^{-1}). The overall minor total growth rates might be a result of long incubation times (49 days) for this experiment, while all incubations with the addition of dispersant (range C6/C8 = 0.002 – 0.004 h^{-1}) led to growth rates lower than the controls ($control_{C6\ /\ C8}$: 0.010 / 0.008 h^{-1}). In consequence, *Cobetia sp.* C6 was picked as the isolate to be investigated further in more detail, as this strain showed slightly better growth rates in comparison to *Cobetia* sp. C8, also with in the dispersed oil incubations.

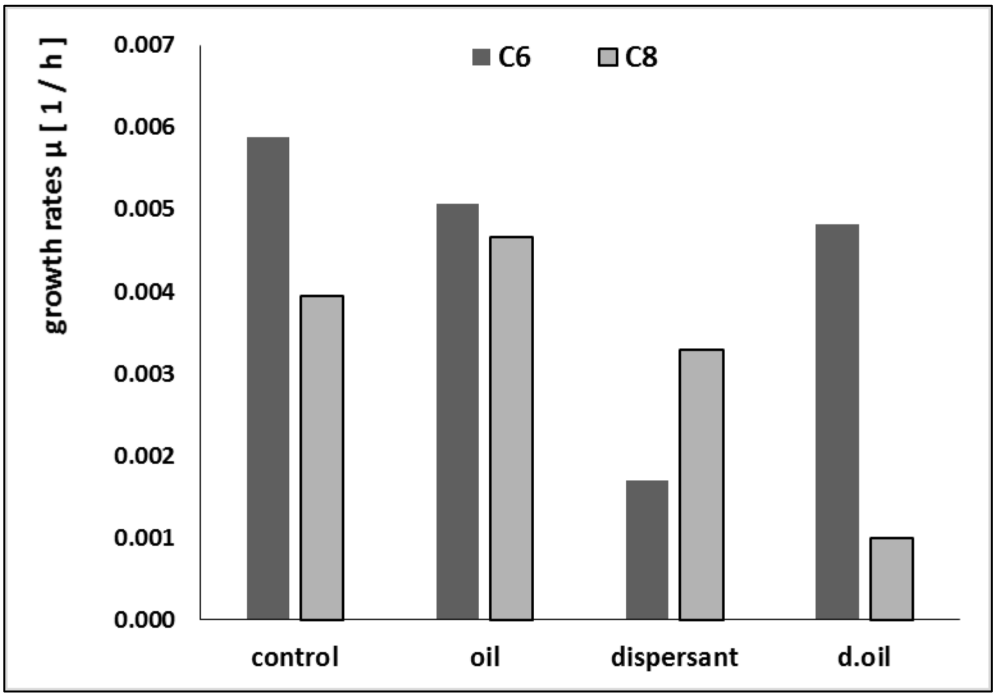


Fig. 3.39: Incubation of Cobetia *sp. C6 / C8 with 30 mL medium (MM2), crude oil ("oil", 1 % v/v, 300 µL), dispersant only ("dispersant", 15 µL) and dispersed oil ("d. oil", 300 µL crude oil, 15 µL dispersant; DOR 1:20) at RT for 1176 h (49 days).*

Substrate preferences of the isolate *Cobetia* sp. C6 towards crude oil hydrocarbons was investigated by a comparison with another complex substrate (lysogenic broth, LB; Fig. 3.40). *Cobetia* sp. C6 incubated with crude oil was able to grow by about one order of magnitude (log CFU/mL(t_{434h}-t_{0h}) = + 0.98 log CFU/mL), while the incubation with LB outgrew this by 2.5 fold (log CFU/mL(t_{434h}-t_{0h}) = + 2.42 log CFU/mL). This indicates that the carbon derived from crude is not the preferred substrate to support the growth of *Cobetia* sp. C6.

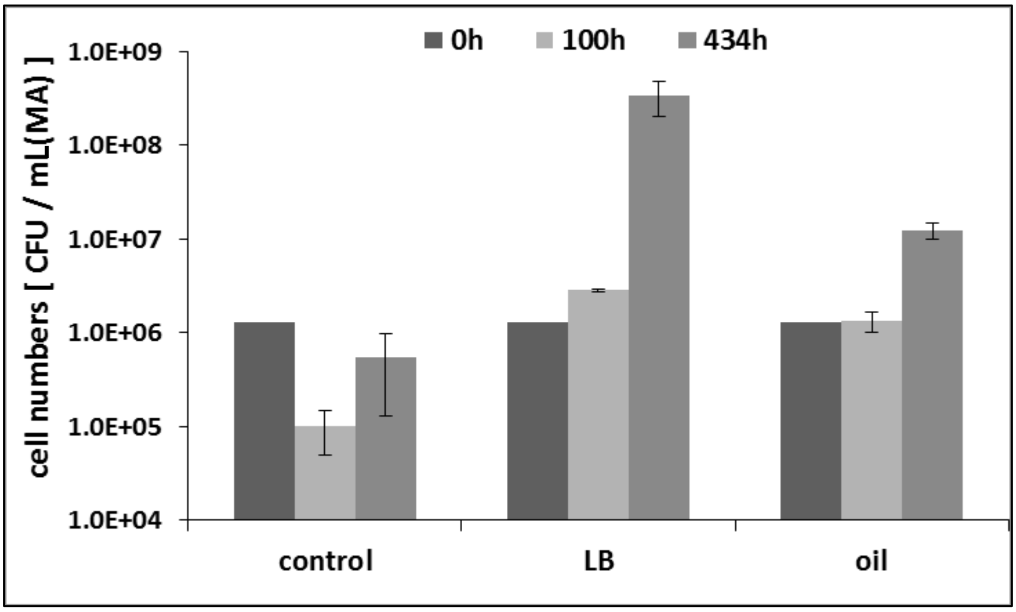


Fig. 3.40: Incubation of Cobetia *sp. C6 in MM2 medium (24 mL) comparing the substrates LB (150 mg) and crude oil (200 µL) at RT for 100 h and 434 h. n = 2.*

Additionally, the effect of temperature on the growth rates of *Cobetia* sp. C6 was investigated (Fig. 3.41), as the strains were isolated from the deep sea sediments of the Gulf of Mexico, where temperatures are relatively constant at 4 °C all year long.

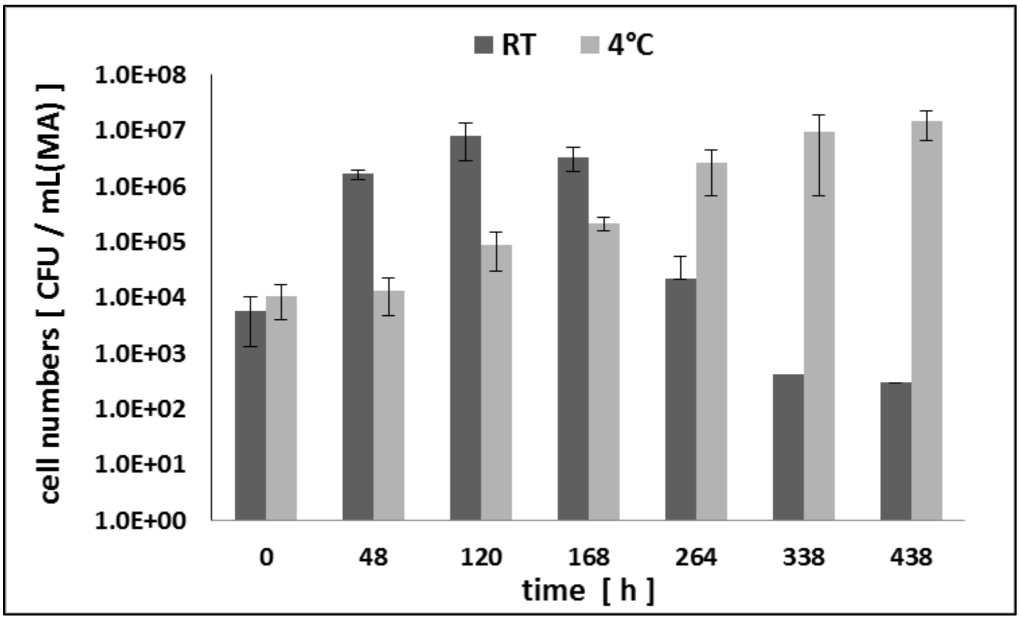


Fig. 3.41: Incubation of Cobetia *sp. C6 in MM2 medium (20 mL) with crude oil (1 % v/v, 200 µL) comparing incubation temperatures (4 °C and RT) for seven timepoints, n = 2.*

Incubations at varying temperatures (RT and 4 °C) showed a difference in growth (Fig. 3.41). Lower temperature did delay the growth of *Cobetia* sp. C6, where the stationary phase (max.

$1.5*10^7$ CFU mL^{-1}) was reached after 264 - 338 hours. At room temperature (RT) growth did already reach the stationary phase after 48 hours (max. $8.1*10^6$ CFU mL^{-1}). Interestingly at room temperature CFU concentrations are decreasing after 168 h again and enter a death phase until the end of the experiment. Unfortunately, it was not possible to observe the similar effect in the 4 °C incubation for the reason of a limited experiment duration.

All isolates (C1 – C8) showed very slow growth rates on crude oil, even in the medium (MM2) with highest nitrogen and phosphorus concentrations, which had been tested. *Cobetia* C6 is not specialized in growth on crude oil components, as peptone and yeast extract led to higher CFU concentrations. A narrow metabolic specialization in single crude oil compounds might have led to a depletion and subsequent limitation in growth in the experiments. However, *Cobetia* sp. C6 grew very slow in all experiments conducted at *in situ* temperature (4 °C), regardless of the substrate (dispersant, crude oil, LB). Higher temperatures (RT), comparable to temperatures at the surface of the ocean, led to an earlier growth.

3.3.3. Growth of *Cobetia* sp. C6 With Crude Oil at Deep Sea Pressure Conditions

Cobetia sp. C6 was isolated from sediment, which was incubated at elevated pressure (10 MPa) for 7 days with crude oil (May17A). The averaged relative abundance of detected *Cobetia* in all experiments was increasing with any incubation in comparison to the starting conditions (t_0 = 0.01 ± 0.02 %; none = 0.07 ± 0.08 %; oil = 0.09 ± 0.18 %; disp = 0.34 ± 0.20 %; d.oil = 0.50 ± 0.57 %). However, there were differences noticeable between the incubations at different pressure conditions, where a higher relative abundance was found at atmospheric pressure (0.1 MPa) in comparison to elevated pressure (10 MPa) (Fig. 3.42). Highest averaged abundance of the genus *Cobetia* was recorded in the dispersed crude oil incubations at 0.1 MPa (0.86 ± 0.60 %).

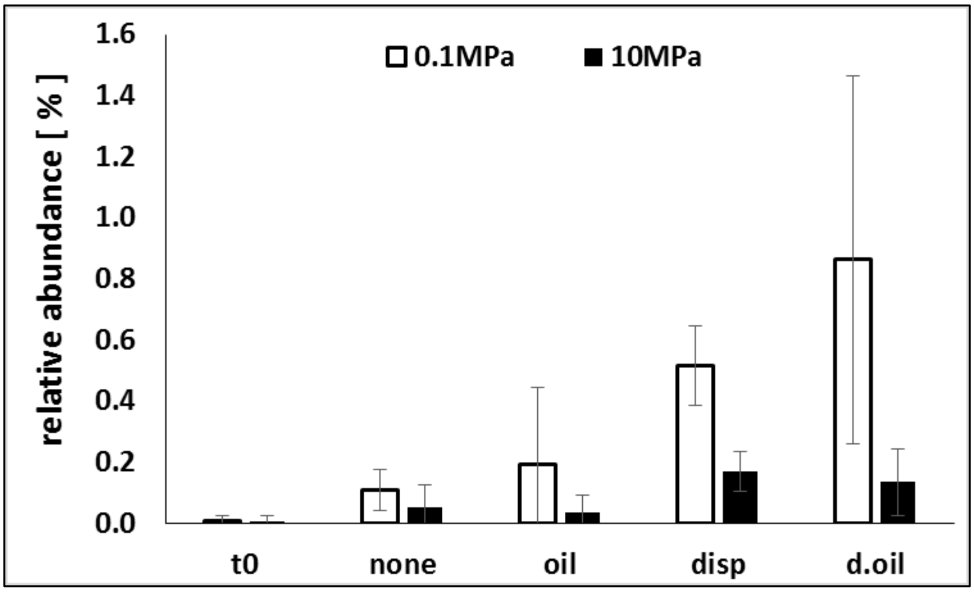


Fig. 3.42: Relative abundances (in %) of genus Cobetia *in all experiments (Okt16, Jan17, May17B, Sept17A, Sept17B, Feb18) before (t_0; n=14) and after incubation with different substrates (none – no substrate (n= 15/23), oil – only crude oil (n = 13/22), disp – dispersant only (n = 6/6), d.oil – dispersed oil (n = 9/9, DOR = 1:100)).*

Incubation of the isolated *Cobetia* sp. C6 at elevated pressure (15 MPa) was conducted to observe the reaction of the single strain with crude oil at different pressures. There was a constant increase in cell numbers in all incubations compared to the start of the experiment (Fig. 3.43).

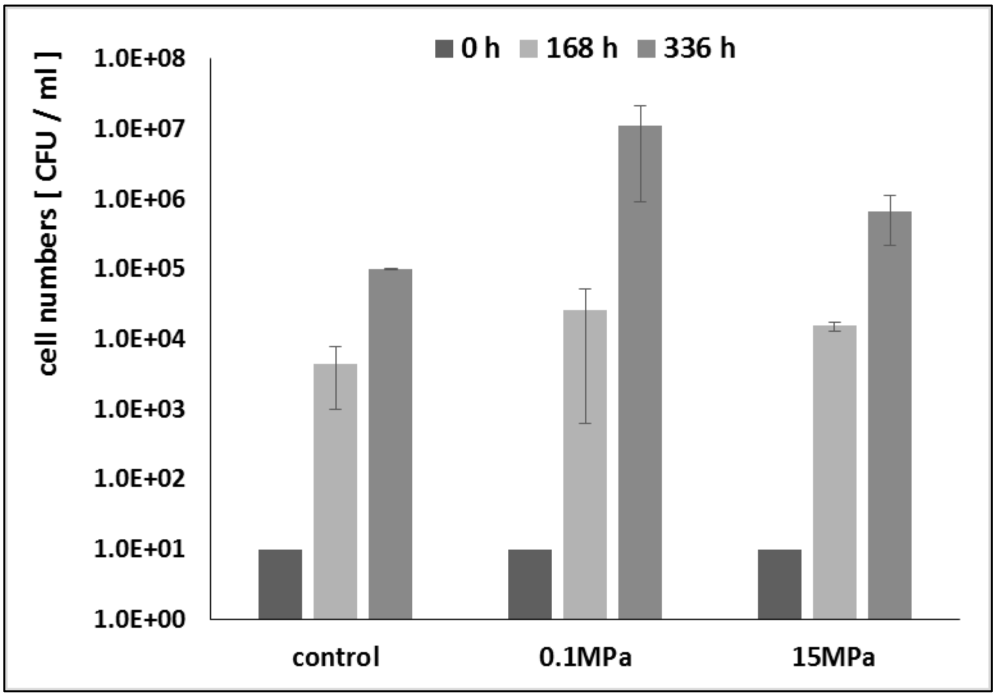


Fig. 3.43: Incubation of Cobetia sp. C6 in MM2 medium (29 mL) with crude oil (1 % v/v = 300 µL) at different pressures (0.1 MPa / 15 MPa) for 168 h and 336 h at 4 °C. n = 2.

Despite the biotic control was able to grow from the initial 10 CFU mL^{-1} to 1.00 ± 0.03*10^{5} CFU mL^{-1} (t$_{336h}$), *Cobetia* sp. C6 grew on crude oil under both pressures. Growth of the control incubation can be explained by remaining oil in the inoculum (1 mL to 29 mL of fresh medium) from the pre-culture. At atmospheric pressure, *Cobetia* sp. C6 grew eventually to 1.13 ± 1.03*10^{7} CFU mL^{-1} (t$_{336h}$), while at elevated pressure to 6.66 ± 4.48*10^{5} CFU mL^{-1} (t$_{336h}$) (Fig. 3.43).

Overall *Cobetia* sp. C6 was able to grow with any pressure applied. Although, the differences between atmospheric and elevated pressure were measureable (Δ cell numbers = 1.06*10^{7} CFU mL^{-1} (t$_{336h}$)), they were statistically not significant ($p = 0.37$). As there were little repetitions ($n = 2$) on this experiment, no conclusion can be drawn from these initial strain characterizing experiments.

3.3.4. Influence of Dispersant and Pressure on the Growth of *Cobetia* sp. C6

To mimic the condition after the DWH incident, a comparison of *Cobetia* sp. C6's behavior at different pressures was extended to incubations with different combinations of dispersant and crude oil added. Two controls were run in parallel: one was the addition of dispersant alone to the incubation (2 µL dispersant to 5 mL of MM2 medium), while to the other only crude oil was added (50 µL to 5 mL of MM2 medium). But the focus of this experiment was the incubation of dispersed crude oil at different pressures. Therefore Corexit EC9500A was added in two different concentrations (0.5 µL and 2 µL, each to 50 µL of crude oil and 5 mL of MM2 medium).

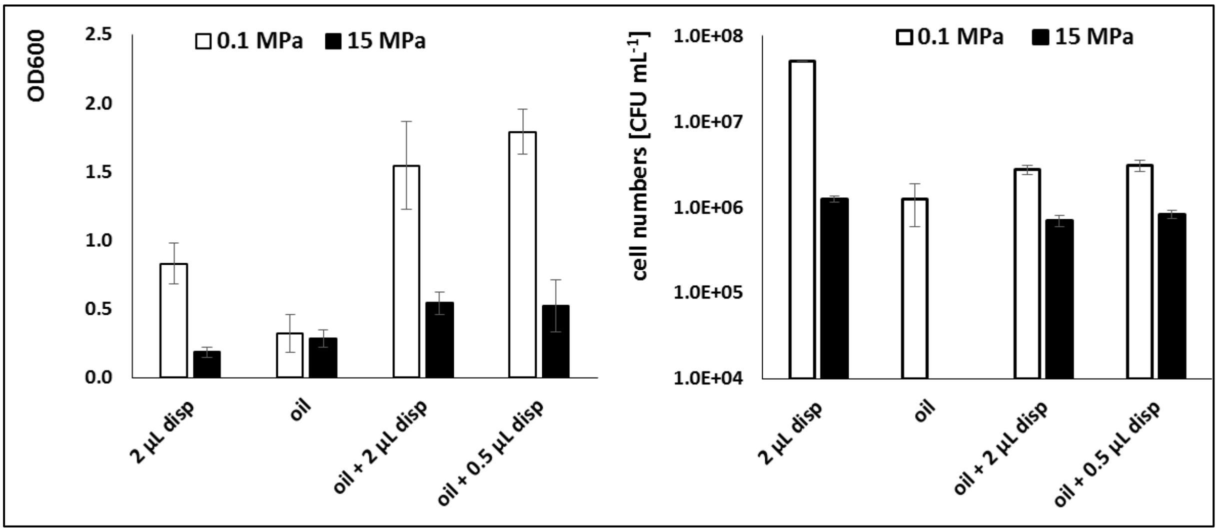


Fig. 3.44: Optical density (left; OD_{600}) and growth rates (right; µ) of the incubation of Cobetia *sp. C6 with different substrates (50 µL crude oil, 0.5 / 2 µL dispersant) at 0.1 and 15 MPa for 480 h at 4 °C and 200 rpm, n = 3. No data could be obtained for the oil only incubation at 15 MPa.*

Growth of *Cobetia* sp. C6 was detected with all added substrate combinations under atmospheric and elevated pressure. OD_{600} values increased substantially in the four dispersed crude oil incubations at both pressures (OD_{600}: 0.294 – 1.980), compared to the rest of the incubations (OD_{600}: 0.132 – 0.977). Partly this is due to the physical dispersion of crude oil, which is interfering with the optical density measurement. However, this affects all incubations likewise and does not explain the high difference in OD_{600} values among dispersed oil incubations at 0.1 MPa and at 15 MPa, 1.667 ± 0.282 and 0.531 ± 0.146, respectively (Fig. 3.44 left). All incubations at atmospheric pressure resulted in higher (averaged) OD_{600} values than the elevated pressure incubations.

The same is true for all growth rate measurements (Fig. 3.44 right). As growth rates are calculated with the time of incubation, obtained values are relatively low for the 20 days incubation. However, even on this low level, growth rates of the atmospheric pressure incubations are consistently higher compared to all elevated pressure incubations. The overall highest value has to be interpreted with caution, as there was just one value for the 0.1 MPa incubation with dispersant only obtained.

For statistical analysis only the incubations with dispersed oil (0.5 µL and 2 µL of dispersant added, n = 12) were taken into consideration, due to less or no values in all other incubations. ANOVA revealed significant differences between the incubations at atmospheric and elevated pressure for OD ($p < 0.001$), CFU mL^{-1} ($p < 0.001$) and growth rates ($p < 0.001$).

As a conclusion from the single cell incubations, it became clear that *Cobetia* sp. C6 is able to grow on dispersed crude oil, as well as on dispersant itself. Further, the influence of pressure is highly relevant for its growth, where elevated pressure is slowing down the growth of this particular strain.

The variants assigned to *Cobetia* of experiment Feb18 were investigated and indicated a similar behavior of *Cobetia* in the community experiments (Fig. 3.45) as the single strain experiments of *Cobetia* sp. C6. Except for the low abundances of the control, the relative abundance of *Cobetia* was always higher (on average) in the atmospheric pressure incubations. This is in accordance with the optical density measurement results of the single strain incubation experiment (Fig. 3.44 left), where dispersed crude oil at atmospheric pressure resulted in the highest abundances. This exceeds the incubations at atmospheric pressure with dispersant and crude oil alone added to the incubation (disp: 0.007 ± 0.003; crude oil: 0.004 ± 0.003; dispersed oil: 0.016 ± 0.006).

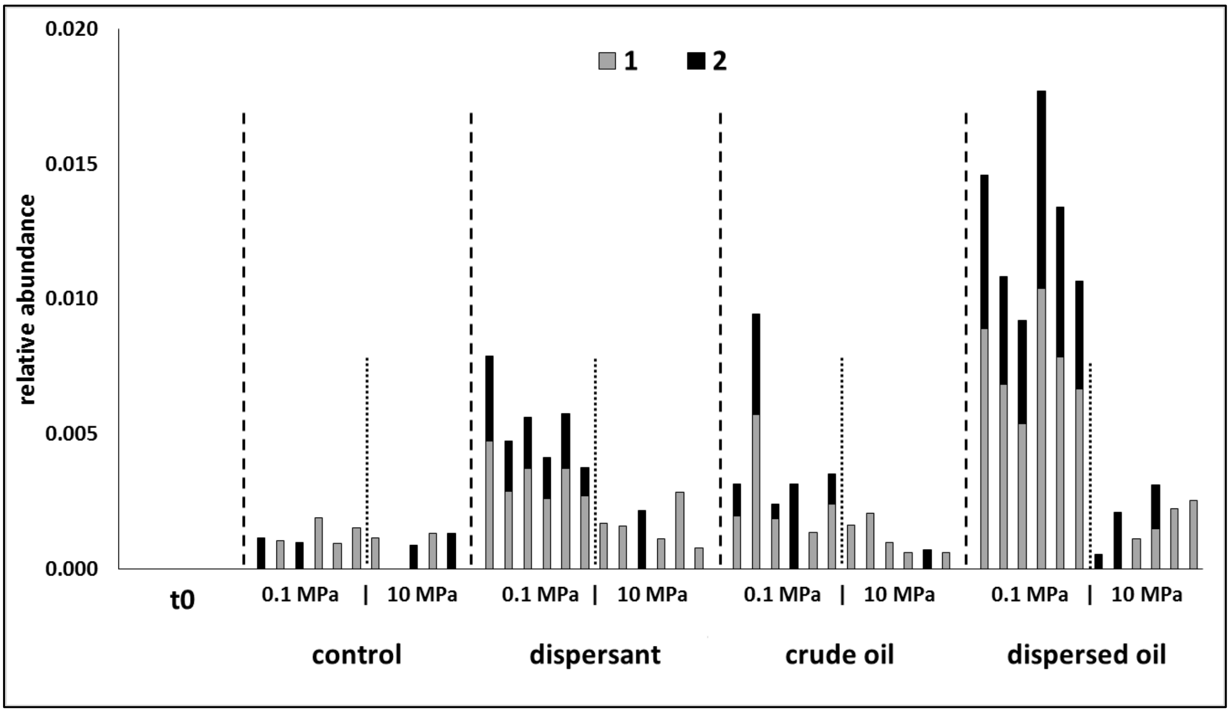


Fig. 3.45: Variant analysis of Cobetia assigned sequences of experiment Feb18 incubations in relative abundances [-]. Sequence variants are indicated with 1 and 2. 5 mL FSW, 50 µL crude oil (1 % v/v), 2 µL dispersant, 4 °C, 168 h, n = 5 – 6, 200 rpm.

The partial 16S rRNA sequences of the two variants were blasted against the NCBI database and first hits were *Cobetia marina* strain 13-5 (Acc.-Nr.: MH037309; 100 % sequence identity; Hassanshahian,M. et al; Isolation and Characterization of crude oil degrading bacteria from some sponges at Persian Gulf) for variant 1 and *Cobetia* sp. R2-Sid-T25-14 (Acc.-Nr.: HF912783; 100 % sequence identity; Kaesler-Neumann,I.; Investigation of sponge-associated bacteria from marine cold-water sponges) for variant 2.

In conclusion, pressure had an effect on the growth of *Cobetia* C6 (Fig. 3.44). *Cobetia* C6 grew best on dispersant and dispersed oil at atmospheric pressure. At elevated pressure (15 MPa) growth with any substrate added was inhibited. This is in accordance with the detected *Cobetia* sequence variants in the sediment experiments, where relative abundance was highest with dispersed oil at 0.1 MPa. Summarizing *Cobetia* sp. C6 can be considered a crude oil and dispersant degrading strain, whose growth is inhibited by elevated pressure.

3.3.5. Error Analysis

The methods to quantify growth of *Cobetia* spp. used in this chapter (3.3) are similar to those, which had been used in the first approach (3.1). Therefore the sources of error in using culture dependent methods have already been described in chapter 3.1.6, e.g. pipetting of viscous substrate, selectivity of growth medium. While the methods, and the respective sources of error, during the analysis of *Cobetia* sequences obtained in sediment experiments have also been discussed before (3.2.8).

4. Overall Discussion and Outlook

Ecosystems are networks, which drive the global energy and element cycle. The largest habitat, by area and biomass on earth is the deep sea (Jørgensen and Boetius, 2007), where the vast majority of biomass and diversity are prokaryotic live forms (Wasmund et al., 2017).
As deep sea drilling for crude oil is continued (Kennicutt, 2017), the probability for the deep sea of being exposed to accidentally spilled crude oil increases. The response of an underexplored ecosystem to these perturbations is of interest to scientists, emergency responders and policy makers.
Obtaining insight into the response of the deep sea bacterial community to the immense input of carbon and energy sources to an otherwise oligotrophic environment was the aim of this thesis. Beside the reaction to crude oil, hydrostatic pressure and the use of a dispersing agent were investigated as environmental influences on the bacterial community and on single representatives of the deep sea prokaryotic life.

4.1. Single Strain Approach

The strain *Rhodococcus* sp. PC20 had been isolated from the GoM deep sea sediment with crude oil at atmospheric pressure. The strains ability to become dominant in the incubations, already indicates its tolerance to different pressures and proofs its competitive fast growth on crude oil. *Rhodococcus* sp. PC20 was not able to grow on aromatic compounds (Fig. 3.5). Even though there were differences in single *n*-alkanes, the strain displayed no substrate specificity or general trend in the preference for alkanes with certain carbon number (Fig. 3.6). *Rhodococcus* species are known to possess multiple alkane hydroxylases (De Carvalho & Da Fonseca, 2005) (Van Beilen, et al., 2002) which were proved to degrade multiple n-alkanes. Therefore *Rhodococcus* sp. PC20 can clearly be considered a generalist, which was confirmed by the fast growth and high cell concentration with lysogenic broth (LB), even exceeding the growth on crude oil. Taking the applied pressures (0.1 and 15 MPa) into consideration, *Rhodococcus* sp. PC20 can be considered a piezotolerant strain, with competitive growth rates on crude oil. Dispersant, even at low concentrations, resulted in delay or inhibition of growth. This negative effect increased with elevated pressure.

This toxic effect of dispersant on the abundance of single hydrocarbon degrading bacteria is known (Hamdan and Fulmer, 2011) and reported to be species-specific (Overholt et al., 2016). As pressure (Fig. 3.7) and the amount of oil (Fig. 3.3) did not influence the strain's growth ability, the inhibiting effect of dispersant most likely led to missing references of *Rhodococcus* among the thriving genera after the DWH spill.

4.2. Community Approach

It had been estimated, that the bacterial community was responsible for at least 43 % of the degradation of the spilled crude oil after the DWH (Joye, 2015). Consequently there is a high interest, how this important entity is affected by environmental factors like crude oil, pressure, and dispersant. Several *ex situ* studies investigated the responses of the deep sea communities to the spilled crude oil and dispersant after the DWH incident (Baelum et al., 2012, Kleindienst et al., 2015, Techtmann et al., 2017). Different approaches investigated the bacterial community at elevated pressure and found a reduced activity (Nguyen et al., 2018), and a synergistic effect of elevated pressure (35 MPa) and low temperatures (4°C) on the bacterial community (Marietou et al., 2018).

The absolute abundance (CFU, Fig. 3.18) increased with incubation for any period. Substrate had a significant influence on the CFU, while pressure did not. The typical decrease in cell numbers with depth (Schwarz et al., 1975, Marietou et al., 2018) was not observed, either due to insignificant pressure application or methodical biases.

The rationale for this approach was to incubate the sediment surface community, as it was supposed to inhabit settled water column organisms. This was partly realized, as typical deep sea water column taxa increased in relative abundance without any substrate added (Fig. 3.17; Orcutt et al. 2011). However, the relative abundances hardly resemble *in situ* bacterial community compositions (Fig. 3.22, Hazen et al., 2010).

Mainly Alteromonadales and Oceanospirillales had been found to respond in the deep sea to hydrocarbon input (Scoma et al., 2016), which is confirmed by the findings, that nearly all differentially abundant taxa belonged to those two orders dealt with in this study (Tab. 3.2). A major phenomenon of the hydrocarbon input to the deep sea, was the drop in diversity (Mason et al., 2012), which could be replicated in the high pressure reactors (Fig. 3.21). Rivers (2013) reported that 95% of the 16S rRNA gene sequences in their samples were from only four families: Colwelliaceae, Oceanospirillaceae, and Piscirickettsiaceae, Methylococcaceae. In this study Colwelliaceae, Oceanospirillaceae and additionally Shewanellaceae made up 76.2 % of all 16S sequence reads (all t > 0 incubations, Feb18).

Alteromonadales, including *Colwellia* and members of the Shewanellaceae, were enriched in unamended long-term pressurization incubations and *Colwellia* was suggested to increase in abundance in response to environmental perturbation (Peoples et al., 2019, Case et al., 2015). The increase in relative abundance of *Colwellia* and Shewanellaceae in all treatments and controls from initially diverse communities suggests that these genera responded to incubation conditions and might have grown on the existing dissolved organic materials without additional hydrocarbon supply.

Statistically, pressure and substrate addition had a significant influence on the bacterial community composition (Fig. 3.30 and Fig. 3.32). A significant increase in relative abundance was only observed for the genera *Moritella* and *Shewanella* and one *unidentified* Oceanospirillaceae genus with pressure and substrate addition. The majority of taxa decreased in relative abundance (66 %) and decreased further when dispersant was present and overall responses were more drastic with dispersant or dispersed oil (Suppl.Tab.4) in comparison to the control incubation. Among the benefiting taxa, the known deep sea genus *Moritella* was clearly responding to the input of dispersant at atmospheric pressure. This is astonishing, as this genus is rarely mentioned in the literature about dispersed oil degradation. Other genera did not display such a clear trend in their behavior to pressure or substrate and it was difficult to separate influences of substrate and pressure.

This stresses the fact, that abundance and activity of a taxon do not have to be linked. Ability to thrive on certain substrates might vary on a sub-genus or even sub-species level (Delmont and Eren, 2016, Kleindienst et al., 2016b). These levels are often referred to as ecotypes, which accounts for the fact, that organisms belonging to the same species might be adapted to different environmental conditions. To gain further knowledge, variant analysis was performed and different behavior of variants assigned to the same representative sequence (Fig. 3.29, Fig. 3.34) were found. Just a few of the significantly different abundant variants were observed to have a peak in relative abundance in the dispersed oil incubation (d.oil) at 10 MPa (*Shewanella* v_3, *Colwellia* v_3 and v_4). *Colwellia* v_3 and v_4 shared a 100 % 16S identity with sequences submitted by Yang,T., Speare,K., Teske,A. and Joye,S. investigating the bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater

Horizon blowout. The responses to the DWH spill might promote single ecotypes, which are specialized in degradation of crude oil components under specific environmental conditions, taking also pressure into consideration.

In the attempt to rank the applied influencing factors, only a small proportion (16 %) of the variances can be explained by the model (Tab. 3.3), which is not unlikely for environmental systems with complex interactions and influences. In a recent study on the microbiom of mite infected foxes contributing to tumor growth, the OLS model gave similar low coefficient of determination values ($R^2 = 0.092$, DeCandia and King, 2019). Besides that, dispersant possesses the highest explanatory power (9 %) among the factors investigated in this study's OLS model. Pressure accounts for 5 % of the variances. In comparison to the average depth of the oceans (3000 m) a relative mild pressure (10 MPa) was applied in these experiments. This strongly recommends to take pressure into consideration and implement it in studies on deep sea hydrocarbon degradation. Thereby the immense influence of temperature (Fig. 4.1, Liu et al., 2017) might have to share some of its importance as an influencing factor with pressure and would leave much space for dispersant.

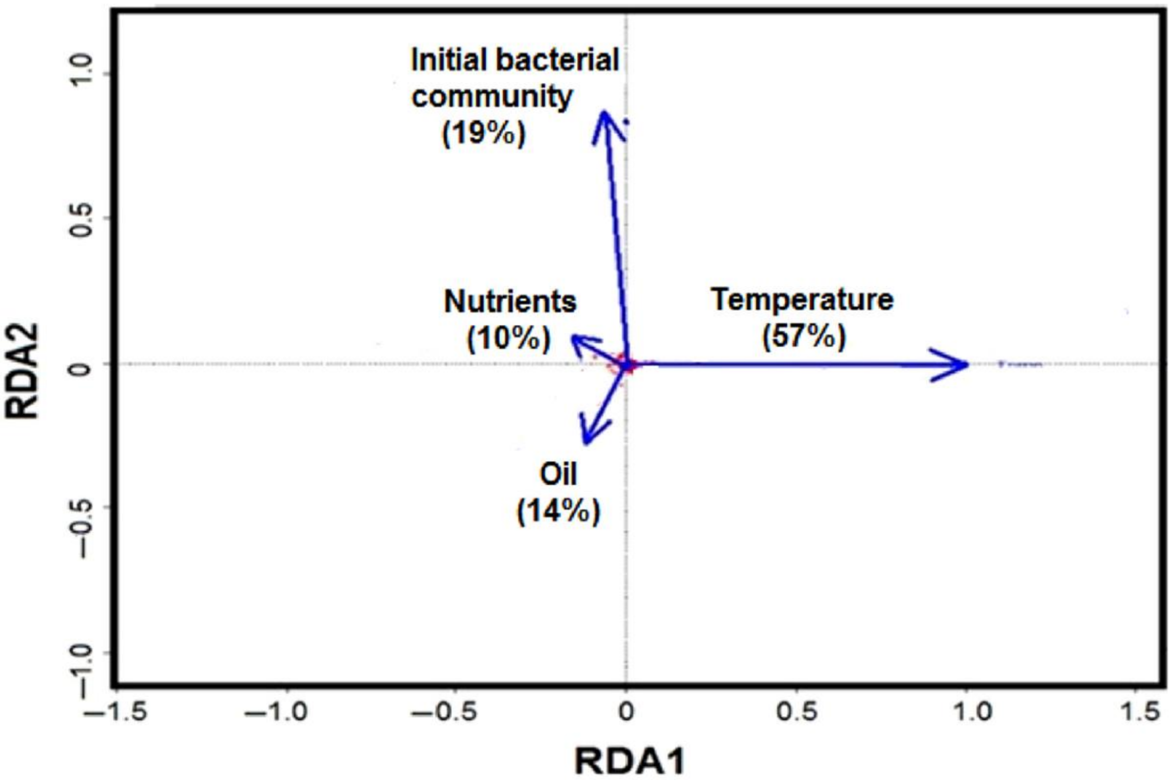


Fig. 4.1: Adapted from Liu et al., 2017 and Bacosa et al., 2018. Biplot diagram of the redundancy analysis (RDA) on microbial community constrained by the presence of oil, temperature, nutrients and initial community.

4.3. Isolation Approach

One of the drawbacks of next generation sequencing (NGS) methods is that identity and function are not connected directly and only the abundance of a taxon does not represent e.g. its ability to degrade certain substances. It might also be the case that this particular taxon lives highly efficiently on the byproducts of one or more less abundant taxa.
Therefore, the isolation of a hydrocarbon degrader following a community experiment was conducted. All applied selective factors in this attempt led to the isolation of a representative of the Oceanospirillales family: *Cobetia* sp. C6 and enabled quantitative growth experiments.

This genus was low abundant in all community experiments, but able to grow at any pressure (0.1 and 15 MPa) and at any substrate. However, *Cobetia* sp. C6 thrived best on dispersant at atmospheric pressure (Fig. 3.43 and Fig. 3.44), which confirms the relative abundances detected in Feb18 experiment (Fig. 3.45). This can be considered a successful attempt to "extract" one genus from the whole bacterial community and investigate its ability to grow on the substrates amended and the conditions applied.

4.4. Overall Conclusion

Two different methods, culture-dependent (plating) and culture-independent (NGS), were applied in this study in order to explore the hydrocarbon degrading bacterial communities in the deep sea. Both methods have their disadvantages and advantages. Culture-independent methods are relative measurements and susceptible to methodical biases (e.g. primers). Culture-dependent methods are highly selective and the information gained is limited. Therefore the two methods were combined in two different ways:

The first one was to isolate a promising representative (*Rhodococcus* sp. PC20) from the sediment of the GoM and compare its abundances and performance to incubations with whole sediment microbial community. As *Rhodococcus* spp. were not identified among the highly abundant taxa in the *in situ* bacterial community analysis, we were able to identify dispersant as an inhibitor to the otherwise highly efficient crude oil degrading strain.

The second approach was to initially perform sediment incubations and start isolation procedures from these incubations after the experiments were finished. The expectation was to isolate a hydrocarbon degrader, which became dominant in the incubations. We could show here, that the performance of the isolated strain (*Cobetia* sp. C6) could be compared to the abundances of sequences assigned to the genus *Cobetia* in the community. This enabled further investigations on the strain's requirements and behavior in a confined space.

This study could show that a combination of "old" and "new" methods potentiates the abilities to investigate a complex system like the hydrocarbon degrading community of the deep sea. This proceeding could help in revealing stepwise the ways how pressure and dispersant influence the bacterial community.

4.5. Outlook

This study reveals the influence of pressure and dispersant on the deep sea bacterial community composition and stresses the fact that pressure should be taken into consideration in future experiments. Obtaining in parallel information on absolute cell numbers, as well as the degradation potential of the bacterial community in all conducted experiments would help complete the picture of crude oil degradation in the deep sea. Possible methodical attempts could be a crude oil analysis by GC-MS and cell quantification via flow cytometry or fluorescence microscopy.

Further, an application of different pressures and extending these pressures matching current drilling depths would give valuable detailed insights into pressure effects, especially in combination with transcriptomic analysis of a single strain. With little effort analysis of further marker genes other than 16S (e.g. *alkB*) on the already extracted DNA would help to obtain insight into the metabolic potential of the incubations.

A comparison of the efficiency between dispersant and the addition of one or more degradation rate limiting nutrient sources (biostimulation/fertilization, Atlas, 1995, Nikolopoulou and Kalogerakis, 2018) under deep sea conditions would help evaluate response strategies in the future.

5. Summary

Deep sea oil spills, like the catastrophe of the Deepwater Horizon (DWH) platform, are a historical novelty in the oil spill history. Technical challenges to the crude oil remediation originate from the depth of the well situated in the deep sea and led to a constant flow over a period of 87 days. One measure to recover the spilled oil was the use of a dispersing agent, Correxit 9500A, which forms smaller oil droplets. The ultimate fate of crude oil in the oceans is the biodegradation by microorganisms, where especially several bacterial taxa are known to degrade crude oil effectively. The effects of elevated hydrostatic pressure typical for the deep sea and the use of dispersant added to crude oil on hydrocarbon degrading single strains and the whole microbial community was investigated in this study in high pressure reactors.

Investigations on a single strain isolated from the Gulf of Mexico's (GoM) deep sea sediments (1500 m bsl.) showed a tolerance towards the tested *in-situ* pressure of 15 MPa compared to atmospheric pressure (0.1 MPa). On the other hand, a ratio of dispersant to crude oil of 1:100 (and higher) inhibited growth of the isolated *Rhodococcus* sp. PC20 strain at any pressure.

The incubation of GoM sediment revealed changes in the bacterial community due to the applied environmental factors pressure, crude oil and dispersant. Most studies on the bacterial reaction to crude oil and dispersant addition neglected the pressure of the deep sea so far. Our results showed significant changes of the bacterial community to pressure and to the addition of crude oil and dispersant. Of these, dispersant was the most influential driving force on the changes in the relative abundance of the bacterial community.

Another single strain, *Cobetia* sp. C6, was isolated from incubations with crude oil at 10 MPa, but showed best growth in incubations at atmospheric pressure and the addition of dispersant, either alone or in combination with crude oil.

This study showed that hydrostatic pressure is a vital environmental factor influencing the crude oil biodegradation potential of deep sea organisms. In addition to this, the importance of dispersant to the relative abundance of the microbial community and to the effectiveness of single crude oil degrading bacteria could be demonstrated.

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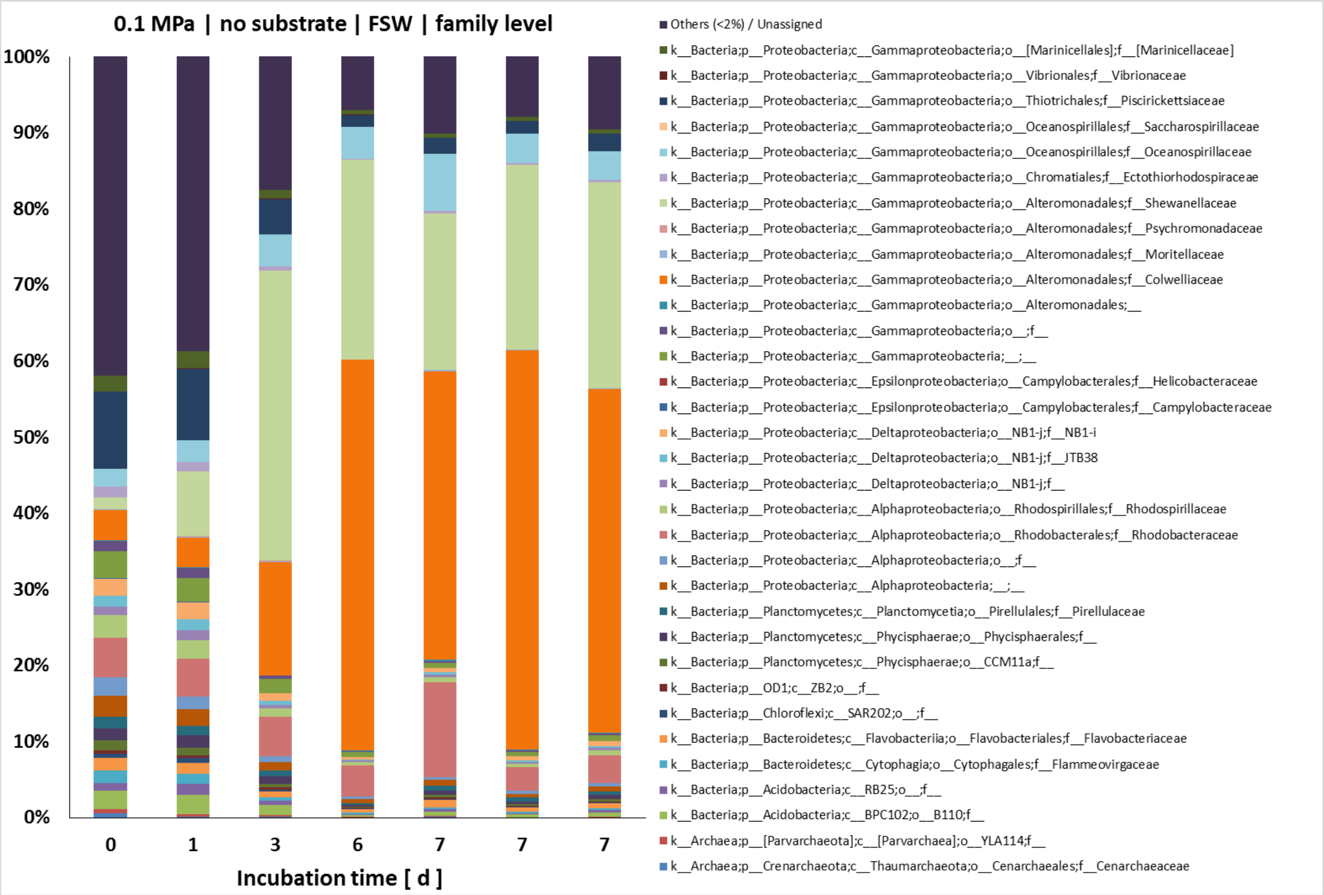
7. Supplementary Material

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value
3.10	**9.1**	14	999	2.888	0.002
3.10	**Feb18**	61	999	6.637	0.001
3.10	**May17B**	21	999	4.354	0.001
3.10	**Sept17A**	29	999	5.774	0.001
3.10	**Sept17B**	36	999	6.672	0.001
9.1	**Feb18**	57	999	4.843	0.001
9.1	**May17B**	17	999	1.771	0.002
9.1	**Sept17A**	25	999	2.089	0.001
9.1	**Sept17B**	32	999	2.008	0.001
Feb18	**May17B**	64	999	4.876	0.001
Feb18	**Sept17A**	72	999	9.805	0.001
Feb18	**Sept17B**	79	999	12.350	0.001
May17B	**Sept17A**	32	999	1.752	0.002
May17B	**Sept17B**	39	999	2.258	0.002
Sept17A	**Sept17B**	47	999	2.137	0.002

Suppl.Tab.1: Pairwise PERMANOVA of all experiments, including all samples.



Suppl.Fig.1: Relative abundances on a family level from three experiment (Okt16, Jan17, May17B) without any substrate for 7, 15, 28, 56 days in artificial seawater at elevated (0.1 MPa) pressure.

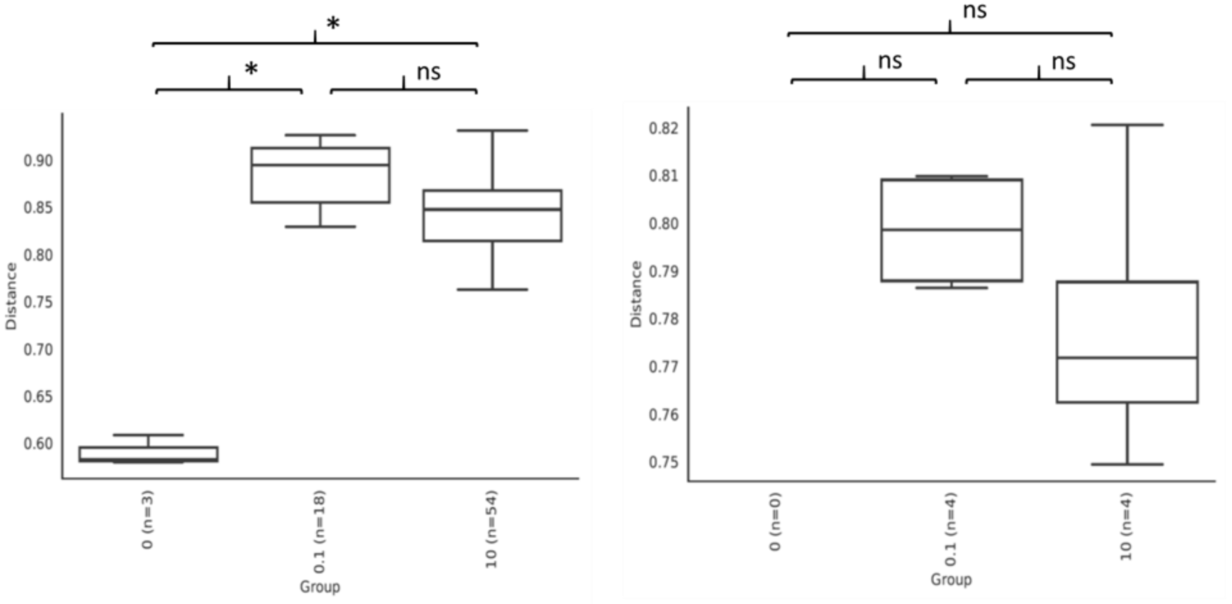
	SeptB							
	t0 (n = 3)				incubation (n = 18)			
	average	SD	min	max	average	SD	min	max
Acidobacteria;c__BPC102	0.027	0.004	0.023	0.033	0.005	0.002	0.001	0.009
OD1;c__ZB2	0.017	0.002	0.015	0.020	0.006	0.002	0.002	0.008
Planctomycetes;c__OM190	0.026	0.004	0.023	0.031	0.005	0.002	0.001	0.009
Planctomycetes;c__Phycisphaerae	0.076	0.004	0.071	0.082	0.018	0.007	0.007	0.031
Planctomycetes;c__Planctomycetia	0.022	0.001	0.021	0.023	0.009	0.003	0.004	0.015
Proteobacteria;c__Alphaproteobacteria	0.091	0.010	0.079	0.103	0.028	0.006	0.016	0.038
Proteobacteria;c__Deltaproteobacteria	0.143	0.002	0.140	0.144	0.029	0.010	0.013	0.055
Proteobacteria;c__Epsilonproteobacteria	0.017	0.001	0.015	0.019	0.014	0.016	0.004	0.059
Proteobacteria;c__Gammaproteobacteria	0.218	0.004	0.213	0.222	0.807	0.059	0.682	0.899
WS3;c__PRR-12	0.021	0.003	0.017	0.023	0.004	0.002	0.001	0.008
others	0.341	0.005	0.334	0.346	0.077	0.027	0.037	0.125

	Feb 18							
	t0 (n = 6)				incubation (n = 47)			
	average	SD	min	max	average	SD	min	max
Acidobacteria;c__BPC102	0.029	0.003	0.026	0.034	0.004	0.002	0.000	0.008
Acidobacteria;c__RB25	0.020	0.006	0.013	0.031	0.003	0.002	0.000	0.007
Bacteroidetes;c__Cytophagia	0.017	0.003	0.013	0.020	0.003	0.002	0.000	0.006
Chloroflexi;c__SAR202	0.017	0.003	0.012	0.022	0.002	0.002	0.000	0.006
Planctomycetes;c__OM190	0.028	0.003	0.022	0.032	0.005	0.003	0.000	0.014
Planctomycetes;c__Phycisphaerae	0.072	0.007	0.064	0.081	0.013	0.006	0.001	0.027
Planctomycetes;c__Planctomycetia	0.019	0.002	0.015	0.022	0.007	0.002	0.000	0.012
Proteobacteria;c__Alphaproteobacteria	0.097	0.009	0.087	0.114	0.029	0.007	0.011	0.046
Proteobacteria;c__Deltaproteobacteria	0.119	0.010	0.104	0.133	0.020	0.007	0.009	0.040
Proteobacteria;c__Gammaproteobacteria	0.338	0.033	0.291	0.379	0.867	0.048	0.778	0.972
WS3;c__PRR-12	0.013	0.005	0.004	0.020	0.002	0.002	0.000	0.007
others	0.230	0.032	0.181	0.264	0.045	0.020	0.007	0.077

Suppl.Fig.2: Relative abundances of starting conditions (t0) and averaged substrate incubations (incubation) of the experiments Sept17B and Feb18 on phylum level (max. abundance > 0.02).

Suppl.Tab.2: First Hit BLAST (NCBI database) result of sequence variants assigned to Shewanella, Moritella, Colwellia *and unidentified* Colwelliaceae *of experiment Feb18. "GoM" means direct link to GoM source of the first hit sequence.*

VARIANT #	DEFINITION	ACCESSION	IDENTITY	AUTHORS	GoM	TITLE
Shewanella						
3	Uncultured bacterium clone S13T5.6-C6	JN621739	100.00%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
4	Uncultured bacterium clone S13T5.6-A4	JN621752	99.13%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
6	Uncultured bacterium clone EzyYyy24	KX172203	100.00%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
8	Uncultured bacterium clone ST0-G8	JN621488	100.00%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
9	Uncultured bacterium clone EzyYyy24	KX172203	97.85%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
10	Uncultured bacterium clone SP1_26	JF928593	99.13%	Harrison,J.P., Schratzberger,M., Sapp,M. and Osb	no	Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms
12	Uncultured bacterium clone ST0-G11	JN621494	100.00%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
15	Uncultured bacterium clone S13T5.6-A4	JN62175	100.00%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
17	Uncultured bacterium clone S13T5.6-C4	JN621754	100.00%	Vandieken,V., et al	no	Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria
18	Uncultured bacterium clone ExkYyy15	KX173250	100.00%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
Moritella						
3	Uncultured bacterium clone EzlYy229	KX173045	100.00%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
8	Uncultured bacterium clone EzlYy229	KX173045	99.57%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
Colwellia						
2	Uncultured bacterium clone 87V1B87_G11_083	MF571825	100.00%	Probst,A., Dubinsky,E. and Hu,P.	**yes**	Uncultured bacteria 16S rRNA clones from Gulf of Mexico during Deepwater Horizon oil spill
3	Uncultured bacterium clone CzzYy125	KX172708	100.00%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
4	Uncultured bacterium clone EzyYy123	KX172413	100.00%	Yang,T., Speare,K., Teske,A. and Joye,S.	**yes**	Following the oil fallout: bacterial community succession in Gulf of Mexico seafloor sediment after the 2010 Deepwater Horizon blowout
5	Uncultured actinobacterium clone C14	KT336076	100.00%	lothe,M., Wegorzewski,A., Muller,C., Simon,F., K	no	Manganese-Cycling Microbial Communities Inside Deep-Sea Manganese Nodules
9	ammaproteobacteria bacterium P1_100_3AA3	KT907001	100.00%	Farnelid,H.M., Turk-Kubo,K.A. and Zehr,J.P.	no	Identification of Associations between Bacterioplankton and Photosynthetic Picoeukaryotes in Coastal Waters
10	Uncultured bacterium clone Mn13T4_6-B5	JQ863473	100.00%	Vandieken,V. and Thamdrup,B.	no	Identification of acetate-oxidizing bacteria in a coastal marine surface sediment by RNA-stable isotope probing in anoxic slurries and intact cores
Colwelliaceae__						
1	Uncultured bacterium clone OHKB9.62	AB094929	100.00%	Inagaki,F., Suzuki,M., Takai,K., Oida,H., Sakamoto	no	Microbial communities associated with geological horizons in coastal subseafloor sediments from the sea of okhotsk
9	Uncultured bacterium clone EB6_3cmSIP-B26	MK108090	100.00%	Cho,H. and Hyun,J.-H.	no	Microbial communities associated with major terminal electron accepting processes and acetate oxidation revealed by RNA stable-isotope…
10	Alteromonadales bacterium S-2-21	AB550531	99.57%	Enomoto,M., Nakagawa,S. and Sawabe,T.	no	Microbial communities associated with holothurians: presence of unique bacteria in the coelomic fluid
11	Gammaproteobacterium H424	AY370004	99.57%	fanos,K., Harmody,D., Dang,P., Ledger,A., Pompo	no	A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates



Pairwise permanova results (SeptB)						
Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**significance**
0	**0.1**	9	999	12.8	0.010	*
	10	21	999	7.7	0.002	*
0.1	**10**	24	999	1.6	0.132	ns

Pairwise permanova results (MayB)						
Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**significance**
0	**0.1**	5	999	30.6	0.197	ns
	10	5	999	7.4	0.209	ns
0.1	**10**	8	999	1.8	0.141	ns

Suppl.Fig.3: Boxplots of experiments Sept17B and May17B grouped by pressure of incubation. Below Pairwise PERMANOVA on the groups. Group "0" refers to t0, "0.1" to atmospheric pressure (0.1 MPa) and "10" to elevated pressure (10 MPa).

	0.1 MPa			**10 MPa**		
oil	**disp**	**d.oil**	**oil**	**disp**	**d.oil**	
102.0	24.3	35.0	60.3	53.7	52.8	Acidobacteria;c__BPC102;o__B110;f__;g__
134.7	30.9	73.7	78.4	78.1	87.8	Acidobacteria;c__RB25;o__;f__;g__
97.9	30.4	26.5	79.1	69.6	67.6	Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Flammeovirgaceae;g__
86.7	7.6	4.7	53.7	28.1	11.3	Chloroflexi;c__SAR202;o__;f__;g__
74.8	51.7	55.0	76.1	68.4	70.2	Planctomycetes;c__Phycisphaerae;o__CCM11a;f__;g__
80.0	49.7	69.7	77.8	80.0	81.1	Planctomycetes;c__Phycisphaerae;o__Phycisphaerales;f__;g__
79.1	54.4	78.1	91.3	81.1	80.2	Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__
98.0	52.8	32.4	41.4	48.5	77.6	Proteobacteria;c__Alphaproteobacteria;__;__;__
93.1	62.9	77.1	84.9	79.6	77.5	Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodovulum
71.8	30.7	43.8	65.4	53.0	55.6	Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__
167.6	121.2	62.9	17.7	26.7	28.1	Proteobacteria;c__Deltaproteobacteria;o__NB1-j;f__;g__
75.6	96.8	169.2	105.2	72.1	193.1	Proteobacteria;c__Deltaproteobacteria;o__NB1-j;f__JTB38;g__
116.7	46.6	79.2	172.3	127.5	161.0	Proteobacteria;c__Deltaproteobacteria;o__NB1-j;f__NB1-i;g__
88.4	33.5	45.7	43.3	41.4	44.8	Proteobacteria;c__Gammaproteobacteria;__;__;__
91.0	61.1	66.7	71.3	64.2	67.1	Proteobacteria;c__Gammaproteobacteria;o__;f__;g__
83.6	108.8	80.1	101.4	128.2	77.2	Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;__
86.1	77.2	87.8	84.1	71.3	100.6	Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;g__Colwellia
100.9	138.9	116.3	187.3	240.2	94.2	Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;g__Thalassomonas
118.8	1776.7	891.9	378.2	1075.5	468.8	Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Moritellaceae;g__Moritella
121.9	83.1	126.7	141.4	119.1	157.9	Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Shewanellaceae;g__Shewanella
150.3	105.0	199.8	137.7	93.7	175.9	Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;__
101.9	48.1	67.9	61.8	56.5	55.9	Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__
111.3	106.8	91.8	55.6	59.9	73.7	Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Oceanospirillum
101.0	46.9	54.0	84.5	68.2	87.6	Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Piscirickettsiaceae;g__

Suppl.Tab.3: Average percentage of the genera's relative abundance of substrate incubations (crude oil (oil), dispersant (disp), dispersed oil (d.oil)) in comparison to the no substrate control (control$_{0.1/10MPa}$ = 100%) of the most abundant families (> 2% of all sequences) at each pressure. Highlighted numbers for significant (alpha < 0.05, two sided) de/increase (light/dark grey) in abundance (Wilcox-Rank-Sum Test on relative abundance values).

		relative abundance								% of control					
		0.1 MPa				10 MPa				0.1 MPa			10 MPa		
		control	oil	disp	d.oil	control	oil	disp	d.oil	oil	disp	d.oil	oil	disp	d.oil
Acidobacteria	c__BPC102;o__B110	0.005	0.005	0.001	0.002	0.007	0.004	0.004	0.004	2.0	-75.7	-65.0	-39.7	-46.3	-47.2
	c__RB25	0.002	0.003	0.001	0.002	0.004	0.003	0.003	0.004	34.7	-69.1	-26.3	-21.6	-21.9	-12.2
Bacteridetes	f__Flammeovirgaceae	0.004	0.004	0.001	0.001	0.004	0.003	0.003	0.003	-2.1	-69.6	-73.5	-20.9	-30.4	-32.4
Chloroflexi	c__SAR202	0.004	0.003	0.000	0.000	0.006	0.003	0.002	0.001	-13.3	-92.4	-95.3	-46.3	-71.9	-88.7
Planctomycetes	c__Phycisphaerae;o__CCM11a	0.005	0.004	0.003	0.003	0.005	0.004	0.003	0.004	-25.2	-48.3	-45.0	-23.9	-31.6	-29.8
	c__Phycisphaerae;o__Phycisphaerales	0.005	0.004	0.003	0.004	0.006	0.004	0.005	0.005	-20.0	-50.3	-30.3	-22.2	-20.0	-18.9
	c__Planctomycetia;f__Pirellulaceae	0.007	0.005	0.004	0.005	0.005	0.005	0.004	0.004	-20.9	-45.6	-21.9	-8.7	-18.9	-19.8
Alphaproteobacteria	Alphaproteobacteria;__;__;__	0.005	0.005	0.002	0.002	0.006	0.003	0.003	0.005	-2.0	-47.2	-67.6	-58.6	-51.5	-22.4
	f__Rhodobacteraceae;g__Rhodovulum	0.006	0.006	0.004	0.005	0.008	0.006	0.006	0.006	-6.9	-37.1	-22.9	-15.1	-20.4	-22.5
	f__Rhodospirillaceae	0.008	0.005	0.002	0.003	0.007	0.004	0.004	0.004	-28.2	-69.3	-56.2	-34.6	-47.0	-44.4
Deltaproteobacteria	o__NB1-j	0.001	0.002	0.002	0.001	0.003	0.001	0.001	0.001	67.6	21.2	-37.1	-82.3	-73.3	-71.9
	o__NB1-j;f__JTB38	0.002	0.001	0.002	0.003	0.003	0.003	0.002	0.005	-24.4	-3.2	69.2	5.2	-27.9	93.1
	o__NB1-j;f__NB1-i	0.005	0.006	0.002	0.004	0.005	0.008	0.006	0.007	16.7	-53.4	-20.8	72.3	27.5	61.0
Gammaproteobacteria	Gammaproteobacteria;__;__;__	0.010	0.009	0.003	0.004	0.018	0.008	0.008	0.008	-11.6	-66.5	-54.3	-56.7	-58.6	-55.2
	Gammaproteobacteria;g__unidentified	0.003	0.003	0.002	0.002	0.005	0.003	0.003	0.003	-9.0	-38.9	-33.3	-28.7	-35.8	-32.9
	f__Colwelliaceae;__	0.277	0.232	0.302	0.222	0.238	0.241	0.305	0.184	-16.4	8.8	-19.9	1.4	28.2	-22.8
	Colwellia	0.167	0.143	0.129	0.146	0.175	0.147	0.125	0.176	-13.9	-22.8	-12.2	-15.9	-28.7	0.6
	Thalassomonas	0.013	0.013	0.018	0.015	0.006	0.011	0.014	0.006	0.9	38.9	16.3	87.3	140.2	-5.8
	Moritella	0.011	0.013	0.190	0.095	0.004	0.015	0.044	0.019	18.8	1676.7	791.9	278.2	975.5	368.8
	Shewanella	0.260	0.316	0.216	0.329	0.244	0.345	0.291	0.385	21.9	-16.9	26.7	41.4	19.1	57.9
	Oceanospirillaceae;__	0.003	0.004	0.003	0.005	0.006	0.008	0.005	0.010	50.3	5.0	99.8	37.7	-6.3	75.9
	f__Oceanospirillaceae;g__unidentified	0.026	0.027	0.013	0.018	0.048	0.029	0.027	0.027	1.9	-51.9	-32.1	-38.2	-43.5	-44.1
	Oceanospirillum	0.004	0.005	0.005	0.004	0.009	0.005	0.005	0.006	11.3	6.8	-8.2	-44.4	-40.1	-26.3
	Piscirickettsiaceae;g__unidentified	0.023	0.023	0.011	0.013	0.030	0.025	0.020	0.026	1.0	-53.1	-46.0	-15.5	-31.8	-12.4

Suppl.Tab.4: Average percentage of the genera’s relative abundance of substrate incubations (crude oil (oil), dispersant (disp), dispersed oil (d.oil)) in comparison to the no substrate control (control$_{0.1/10MPa}$ = 100%) of the most abundant families (> 2% of all sequences) at each pressure. Highlighted numbers for de/increase (blue/red) in abundance on a continuous color scale and (absolute) relative abundances for calculations.