Changes in bacterial communities from anaerobic digesters during petroleum hydrocarbon degradation

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A B S T R A C T

Anaerobic biodegradation of petroleum hydrocarbons (PHC) to methane has been recognized to occur in oil reservoirs and contaminated surface sites alike. This process could be employed efficiently for the treatment of contaminated materials, including petrochemical wastes and PHC-contaminated soil, since no external electron acceptor is required. Moreover, the controlled production of methane in digestion plants, similarly to the anaerobic digestion (AD) of energy crops or organic residues, would enable for energy recovery from these wastes. At present, little is known about the bacterial communities involved in and responsible for hydrocarbon fermentation, the initial step in PHC conversion to methane. In the present study, the fate of two different methanogenic communities derived from the AD of wastewater (WWT) and of biowaste, mixed with PHC-contaminated soil (SWT), was monitored during incubation with PHC using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA genes amplified with Bacteria-specific primers. During 11 months of incubation, slight but significant degradation of PHC occurred in both sludges and distinct bacterial communities were developing. In both sludges, Bacteroidetes were found. In addition, in WWT, the bacterial community was found to be dominated by Synergistetes and Proteobacteria, while Firmicutes and unidentified members were abundant in SWT. These results indicate that bacterial communities from anaerobic digesters can adapt to and degrade petroleum hydrocarbons. The decontamination of PHC-containing waste via fermentative treatment appears possible.

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1. Introduction

The release of environmentally harmful petroleum hydrocarbons (PHC) during production, refinement, transportation and storage of crude oil and its products has led to the contamination of considerable areas of land and water around the world. The majority of PHC constituents, including linear (n-), branched (i-) and cyclic alkanes, alkenes and a broad range of aromatic compounds, are well-degradable by microorganisms (Colombo et al., 1996; Heider et al., 1998). Bioremediation aims at utilizing this potential for the clean-up of contaminated matrices via the optimization of degradation conditions. Decontamination options for PHC-polluted soil include microbial treatment in situ and in case the bulk contaminated soil is removed from the site, also ex situ remediation. PHC bioremediation measures mostly focus on the addition of oxygen (Song et al., 1990). Other amendments include nutrients (Bewley et al., 2001; Röling et al., 2002), microorganisms (Tam and Wong, 2008; Van Herwijnen et al., 2006) and other supplements, e.g. to increase contaminant bioavailability (Jimenez and Bartha, 1996; Scherr et al., 2009).

Evidence for the occurrence of microbial crude oil alteration under highly reducing conditions has been accumulating since the 1920s (Bastin et al., 1926). In the past decades, anaerobic PHC biodegradation has eventually been recognized as a process occurring in naturally petroleum-bearing rock as well as in contaminated surface soil and sediment (Delauze et al., 1980; Perry and Gibson, 1977). To date, anaerobic PHC biodegradation with different terminal electron acceptors, including nitrate, sulphate and ferric iron reduction has been described (Hasinger et al., 2012; Siegert et al., 2011; Townsend et al., 2003). In contrast, no separate electron donor, apart from HCO$_3^-$, is required for the conversion of PHC to methane (Conrad, 1999; Jones et al., 2008; Townsend et al., 2003). The role of methane production from PHC, however, is ambivalent. Occurring in the reservoir, it contributes to the deterioration of oil quality (Jones et al., 2008) and methane leakages from reservoirs adversely impact global climate (Milkov, 2011). On the other hand,
methane can be used as fuel and for energy production, and is produced and collected for this purpose via fermentation of energy crops (Lindorfer et al., 2007), organic residues from animal husbandry (Bertin et al., 2008; Resch et al., 2011) and household wastes (Gallert and Winter, 1997), among others. Similarly, the recovery and utilization of methane produced during the anaerobic digestion of petrochemical wastes was proposed as an energetically favorable approach for their disposal (Chou et al., 1978).

Rate and extent of methane production from PHC is expected to be determined by energetic, microbial and toxic constraints. Methanogenic crude oil biodegradation can be assumed to proceed only after the depletion of terminal electron acceptors that are connected to a comparatively higher energy yield. Gibbs free energy, $\Delta G^\circ$, of methanogenesis from n-hexadecane was calculated to be some 29 times lower than for anaerobic oxidation on the expense of nitrate reduction and two times lower than for the sulphate reduction pathway (Heider et al., 1998; Widdel and Rabus, 2001). Analog to the anaerobic digestion of organic residues, the methanogenic degradation of PHC proceeds through the concerted action of several groups of fermenting bacteria and methanogenic archaea (Jones et al., 2008; Zengler et al., 1999). N-alkane fermentation results in the formation of acetate and hydrogen, which are subsequently utilized for methane production via acetoclastic, CO$_2$ reduction and/or syntrophic acetate oxidation (Jones et al., 2008; Zengler et al., 1999). Few microorganisms are known that are operating at the 'top end' of PHC methanogenesis, i.e. that are competent of alkane activation in the absence of electron acceptors other than water/bicarbonate. Evidence has, to our knowledge, only been provided for the genera Syntrophus of the class Deltaproteobacteria (Zengler et al., 1999) and Marinobacter in Gammaproteobacteria (Head et al., 2010). Members of the phyla Firmicutes, Proteobacteria and Bacteroidetes were found to be present at the majority of methanogenic, PHC-contaminated subsurface environments (Gray et al., 2010). Their actual role in PHC fermentation has not been elucidated yet. On the other hand, a possible inhibitory effect by PHC specifically on acetoclastic archaea (Warren et al., 2004) may contribute to limiting methane yield on the 'bottom end' of the process.

Insights into the mechanisms and contributors to microbial conversion of petroleum hydrocarbons to methane contribute to the assessment of the feasibility of decontamination and energy recovery from PHC-contaminated solid waste in anaerobic digesters. As a first step towards possible application, the objective of the present study was to characterize the effect of PHC addition on different anaerobic digester communities and their potential to degrade petroleum hydrocarbons.

In laboratory-scale microcosms, bacterial population changes and concurrent PHC degradation in two unadapted methanogenic sludges were studied. The sludges were obtained from anaerobic digestion plants and artificially contaminated with a paraffin-rich slurry. Sludge WWT was obtained from the digestion tower of a typical communal sewage treatment plant. Sludge SWT was a slurry obtained from a biogas plant digesting solid household and food wastes mixed with 7.5% (w/w) PHC-contaminated anaerobic soil. The soil was obtained from a historically PHC-contaminated industrial site in lower Austria. The soil was a sandy soil according to US Soil Taxonomy and the contamination amounted to approximately 7100 mg kg$^{-1}$ petroleum hydrocarbons (as TPH or total petroleum hydrocarbons). Prior to experimental set up, both sludges and the soil were sieved to <2 mm in order to homogenize them.

To maintain anaerobic conditions, sludge sieving, reactor filling, addition of crude oil and reactor sampling during incubation were performed in an argon-flushed (20 min at 2000 ml min$^{-1}$ with Ar 5.0; Linde Gas GmbH, Stadl-Paura, Austria) anaerobic glove box (Fisher Scientific, Vienna, Austria).

2.2. Crude oil

A paraffin-rich crude oil from the Vienna Basin was used in this study as contaminant for the degradation tests. The GC-FID (gas chromatography with flame ionization detector) chromatogram indicates the prevalence of n-alkanes, appearing as distinct peaks in Fig. 1. Distinct iso- and i-alkanes are also visible, enabling for the calculation of n-alkane/i-alkane area ratios. Changes in these ratios over time are indicative of biodegradation due to the dissimilar degradative behavior of chromatographically ‘neighboring’ methylated and normal alkanes (Bekins et al., 2005; Wang et al., 2007). In this study, ratios of n-C$_{17}$ to 2,6,10-tetramethylpentadecane (pristane, i-C$_{19}$) and of n-C$_{18}$ to 2,6,10,14-tetramethylhexadecane (phytane, i-C$_{20}$) were employed (Fig. 1).

Microbial biodegradation of this crude oil under aerobic (Scherr et al., 2007) and nitrate- and sulphate reducing conditions (Hasinger et al., 2012) is described elsewhere.

2.3. Bioreactor operation and sample collection

Commercially available 500 ml Pyrex$^\circledR$ laboratory glass bottles were used as lab-scale bioreactors. A defined volume of 125 ml of homogenized WWTP or SWT sludges was added to two bottles each. One bottle per sludge parallel was supplemented with a nominal concentration of 25,000 mg kg$^{-1}$ of crude oil (‘degradation test’), the other was left unamended to serve as a blank. After set up, all bottles were flushed with argon for 20 min in order to remove dissolved oxygen and then closed tightly with screw caps equipped with PTFE (polytetrafluoroethylene)-faced sealing discs. Sterile syringes were placed on the bottle caps in order to quantify gas production. The reactor bottles were placed on a multi flask orbital shaker type VKS 75 (Wenk LabTec GmbH, Netetal,
Germany) and incubated at 100 rpm at 35 °C for 325 days in the dark.

After 0, 10, 35, 63, 84, 105, 126, 149, 170 and 325 days of incubation, samples for DNA-analysis were collected from both degradation test and blank bottles with sterile pipette tips in the argon-flushed glove box. Prior to each sampling, reactor bottles were shaken at 1000 rpm for 3 min on a laboratory shaker type IKA-VIBRAX-VXR (Jankel & Kunkel GmbH & Co KG, Germany) for homogenization.

In addition on days 0, 170 and 325, quantitative analysis of volatile fatty acids (VFA) of all parallels and qualitative PHC analysis for the crude oil amended bottles were performed as described below.

2.4. Petroleum hydrocarbon extraction and analysis

Qualitative PHC analysis in slurry samples was performed using chromatographic data obtained following the international standard method DIN ISO DIS 16703 as described previously (Scherr et al., 2007). For sampling, a certain amount of the liquid/oil mixture was collected from the reactors with an ethanol-cleaned stainless steel spatulum. Samples were collected and analyzed at the beginning of the experiment and after 170 and 325 days of incubation, respectively. The area ratios of n-C_{17} to pristane and n-C_{18} to phytane were calculated to detect PHC biodegradation (Wang et al., 2007). For sampling, a certain amount of the liquid/oil mixture was collected from the reactors with an ethanol-cleaned stainless steel spatulum. Samples were collected and analyzed at the beginning of the experiment and after 170 and 325 days of incubation, respectively. The area ratios of n-C_{17} to pristane and n-C_{18} to phytane were calculated to detect PHC biodegradation (Wang et al., 2007). These peak areas were integrated separately.

To allow gel-to-gel comparison of different samples, a reference DNA sample was used.

2.5. Analysis of volatile fatty acids (VFA), sulphate and nitrate

Samples obtained from the bioreactors were subject to analysis of typical fermentation products from anaerobic digestion including short chain fatty acids, sugars and alcohols. These comprised acetic, propionic, butyric, lactic, formic, galacturonic, valerianic and iso-valerianic acids, lactose, glucose, fructose and 1,3- and 2-propanediol, glycerol and ethanol. These were determined on an Agilent HPLC Series 1100 (Agilent, Austria) equipped with an RI detector in a calibration range from approximately 0.1 to 1000 mg l^{-1} as described before (Scherr et al., in press). Sulphate and nitrate were analyzed using ion chromatography as described elsewhere (Scherr et al., in press).

2.6. DNA extraction and PCR amplification

Total genomic DNA was extracted from the sludges according to Klocke et al. (2007). DNA yield was visually evaluated by comparison with a DNA standard after electrophoresis in 0.8% agarose gel. 16S rDNA fragments corresponding to nucleotide positions 341–926 of the Escherichia coli 16S rDNA numbering system covering variable V3 and V4 regions were amplified with the bacteria-specific forward primer 341F-GC (Muyzer et al., 1993) and the reverse primer 907r (Muyzer et al., 1999). The PCR was performed in a Biometra T-personal thermocycler (Whatman Biometra, Göttingen, Germany) and the presence of PCR products was confirmed by analyzing 5 μl of product by electrophoresis in 1% agarose gels and SYBR® Safe DNA gel staining (Invitrogen, Eugene, Oregon, USA) and UV light prior to subsequent DGGE.

2.7. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with a Dcode System apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instruction. Amplicons of approximately 560 bp were separated in 6% (w/v) polyacrylamide gels containing a linear 35–65% denaturant gradient (100% denaturant corresponds to 7 M urea and 40% deionized formamide). Electrophoresis was performed in 1 x tris-acetate-EDTA (TAE) buffer at a constant voltage of 100 V and a temperature of 60 °C for 16 h (Gerdes et al., 2005). After completion of electrophoresis, the DNA bands were stained by using 0.01% SYBR® Green I nucleic acid gel stain (Sigma–Aldrich Chemie GmbH, Germany; Cat. No. S9430) in 1 x TAE buffer (pH 8.0) and were photographed with a Typhoon Trio™ Variable Mode Imager (Amersham Biosciences, Sunnyvale, CA) at 488 nm wavelength. To allow gel-to-gel comparison of different samples, a reference marker containing different 16S rDNA PCR products was used.

2.8. Band excision, reamplification and sequencing

Each gel slice that contained an obvious DNA band was excised with a clean scalpel and placed in autoclaved 1.5 ml centrifuge tubes. The slices were crushed with clean pipette tips and incubated with 50 μl of Milli-Q water at 4 °C overnight. The 3 μl supernatant was subjected to a second PCR under the same conditions as described before. The reamplified PCR products were examined by DGGE to confirm that single bands were present at the same position. The PCR products were then purified and concentrated with QIAEX II Gel extraction kit (QIAGEN GmbH, Germany; Cat. No. 20021) and sequenced with the reverse primer 907r by a commercial company (Life Technologies, Foster City, CA). Sequences obtained in this study are deposited in the GenBank nucleotide sequence database under accession numbers JN698223-JN698238 (Table 2).

2.9. Identification of bands by comparative sequence analysis and construction of phylogenetic tree

After editing and trimming manually using BioEdit Sequence Alignment Editor Version 7.0.0 (Hall, 1999), the DNA sequences were searched against the Ribosomal Database Project II (RDP II) release 10.0 (Cole et al., 2007) and the GenBank nucleotide sequence database using the BLAST online searches of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997). All reference sequences were obtained from the GenBank and RDP. Multiple sequence alignments were made with the program CLUSTAL W (Version 1.6) (Thompson et al., 1994). Sequence gaps and ambiguous bases were excluded. Distance matrices were calculated by the two-parameter method of Kimura (1980). The neighbor-joining method was used for constructing phylogenetic trees (Saitou and Nei, 1987). Robustness for individual branches was estimated by bootstrapping with 1000 replications (Felsenstein, 1985) with the program MEGA (Version 4) (Tamura et al., 2007).
and 4.59 to 2.42 (ANOVA/Tukey, 0).

Changes (ANOVA/Tukey post-test, 2005; Head et al., 2010). In the present experiments, no significant manifesting themselves in a commonly show methylated alkanes over incubation time.

Ratios of peak areas of selected normal alkanes to chromatographically neighboring WWT. SWT exhibits a similar PHC pattern.

3.1. Petroleum hydrocarbon biodegradation in SWT and WWT

In the present study, peak area ratios of chromatographically neighboring n- to i-alkanes were calculated to determine the occurrence of PHC biodegradation. Normal alkanes are usually degraded preferentially over their alkylated counterparts of approximately similar equivalent carbon number, i.e. similar chromatographic retention time (Fig. 2; e.g. Bekins et al., 2005; Head et al., 2010). However, preferred degradation of branched over linear alkanes has been noted under specific terminal electron accepting conditions recently (Hasinger et al., 2012). In any case, it can be assumed that closely eluting i- and n-alkanes would be degraded at different rates (Jones et al., 2008), rendering a change in their ratio indicative for the occurrence of biodegradation, assuming no limitation by hydrocarbon bioavailability. In the present experiments, the occurrence of PHC degradation was assessed on the basis of the change in peak area ratios of n-C_{17} to pristane (i-C_{19}) and of n-C_{18} to phytane (i-C_{20}). Results are displayed in Table 1. Underaged oil samples commonly show n-alkane concentrations greater than iso-alkanes, manifesting themselves in a n-:i-peak area ratio >1 (Bekins et al., 2005; Head et al., 2010). In the present experiments, no significant changes (ANOVA/Tukey post-test, p < 0.05, n = 3) were observed for both inocula and both ratios over six months (day 170, data not shown). After 325 days of incubation, in contrast, ratios of n-C_{17}:i-C_{19} and n-C_{18}:i-C_{20} exhibited a significant decrease, from 2.77 and 4.59 to 2.42 (ANOVA/Tukey, p < 0.01) and to 4.16 (p < 0.001), respectively, in WWT and to 2.35 (p < 0.001) and 3.90 (p < 0.001) in SWT, respectively (Table 1). The decreasing specific alkane ratios discussed above are also reflected, on a more general level, in a slight reduction of most n-alkane peaks after 325 days of incubation (Fig. 2, day 325 exemplified for WWT, compared to Fig. 1, day 0).

3.2. Electron acceptors for anaerobic PHC oxidation

It can be assumed that electron acceptors connected to relatively high energy yields (as ΔG°) from their reduction, such as nitrate and sulphate, would be at least partially depleted ahead of the utilization of other, energetically less favorable acceptors, such as HCO_{3}⁻ (Widdel and Rabus, 2001). In the present study, initial concentrations of sulphate amounted to 2.5 and 19.2 mg l⁻¹ and of nitrate to 10.0 and 1.6 mg l⁻¹ in WWT and SWT, respectively. On day 170, nitrate and sulphate were not present in detectable concentrations in any of the parallels, with analytical detection limits of 0.58 and 0.46 mg l⁻¹ for nitrate and sulphate, respectively. This indicates that PHC degradation that was observed in both sludges between incubation days 170 and 325 was occurring in the absence of nitrate and sulphate, potential competitors with HCO_{3}⁻ as electron acceptor in anaerobic PHC oxidation.

3.3. Occurrence of low molecular weight PHC degradation products

Initially, the content of volatile fatty acids in the two inocula was dominated by acetic acid, amounting to 29.5 mg l⁻¹ and 89.7 mg l⁻¹ for WWT and SWT on day 0, respectively. A small amount of gas was produced in all parallels (data not shown) during the first two months of incubation. Since no significant PHC degradation was observed in this time, it appears likely that the residual acetic acid was converted into gas. Concentrations of the analyzed volatile fatty acids, carbohydrates and alcohols were below detection limits (approximately 0.1 mg l⁻¹) for all parallels on days 170 and 325.

In anaerobic digestion (AD), the occurrence and concentration of low molecular weight intermediates are important indicators for the stability of the process (Marchaim and Krause, 1993). The formation of acetic acid represents one of the final steps in methane generation from organic matter and also in the fermentation of crude oil (Jones et al., 2008). In the present experiments the absence of acetic acid in detectable concentrations, despite the occurrence of PHC degradation, can be attributed to different reasons. These include (i) conversion of PHC into gas and/or (ii) accumulation of acetic acid in levels below detectability. Both would correspond to the presumably small, however unquantified, extent of PHC degradation. The hypothesis of acetic acid accumulation would find further support in an inhibition of acetoclastic archaea by PHC such as was observed before (Warren et al., 2004). Compared to other pathways for methanogenesis, archaea responsible for acetoclastic methanogenesis, which dominates in anaerobic digestion (Zinder, 1993), are the most susceptible group to PHC exposure (Warren et al., 2004). However, since the complete removal of acetic acid, adjoined by initial gas production, was observed in all parallels until day 170, a complete inhibition of acetoclastic archaea by PHC can most likely be ruled out. A third reason for the absence of acetic acid may be (iii) the fermentation of PHC to other products such as long chain fatty acids, which were not analyzed presently.

3.4. Microbial contributors to methanogenesis from petroleum hydrocarbons

Bacteria and archaea are considered to play the predominant role in hydrocarbon degradation in an anaerobic environment (Leahy and Colwell, 1990; Widdel and Grundmann, 2010). Fungi are a third type of microorganisms that are commonly associated with hydrocarbon breakdown in soil (Acevedo et al., 2011). In contrast, fungi are not common in AD and rather constitute a sanitary problem in case they do occur (Schnurer and Schnurer, 2006). They are not expected to be key contributors to the anaerobic digestion of PHC.
3.5. Analysis of microbial community shifts

In the present study, shifts in bacterial population patterns are compared between crude oil amended and blank tests, and between the two inocula. Community shifts of the unadapted consortia during exposure to hydrocarbons can be induced by a single mechanism or a combination thereof. In case these shifts are limited to the test bottles supplemented with crude oil, population shifts are likely indicating inhibitory or toxic effects exerted by PHC, bacterial adaptation including induction and expression of existing genes (enzyme synthesis) for PHC digestion and/or an influence on syntrophic bacterial associations in different positions along the anaerobic digestion chain. A similar shifting behavior in both PHC-supplemented and corresponding blank tests, in contrast, would point towards effects not directly related to crude oil addition.

Figs. 3 and 4 display the community shifts associated with incubation of WWT and SWT sludges as is (blanks) and under crude oil amendment over a time of 325 days, as detected with PCR-DGGE fingerprints of the 16S rDNA gene. Table 2 presents selected bands from DGGE fingerprints as well as their closest matching sequences from the GenBank nucleotide database and the accession numbers. In total, 11 and 12 different 16S rDNA fragments were sequenced from the WWT and SWT sludge samples, respectively, matching with the majority of intense bands and also with several faint bands of the fingerprints. Sequence analyses revealed that they were phylogenetically affiliated to sequences belonging to members of the phyla Betaproteobacteria, Bacteroidetes, Synergistetes and Firmicutes. Identification at the genus or species level could not be obtained. A high number of sequences were affiliated to unidentified bacterial sequences in the range of 73–99% similarity (Table 2). Members of these four phyla have been reported previously as inhabitants of hydrocarbon containing environments, subsurface oil reservoirs (Orphan et al., 2000; Vasconcellos et al., 2010), soils and sediments (Gray et al., 2010) as well as anaerobic digesters (Huang et al., 2004; Nelson et al., 2011). This underlines the ability of these microorganisms to thrive on a variety of substrates as well as their resistance to hydrocarbon toxicity. Their role in hydrocarbon degradation under methanogenic conditions has, however, not been characterized.

A phylogenetic tree was constructed by the neighbor-joining method with the reference 16S rDNA sequences obtained from the GenBank database (Fig. 5). The tree reflects the results from sequence alignment and also provides additional information about taxonomic affiliations of the sequences which remained unidentified with BLAST search.

3.6. Community shift in wastewater treatment plant sludge (WWT)

Both WWT sludge parallels show numerous dominant and faint bands which are present throughout incubation time (Fig. 3). Table 2 shows the results of sequence analysis at the phylum level. Bacteria corresponding to bands 2214 and 2216–2218, present in both parallels over the entire incubation time, were found to be members of the phylum Synergistetes. Synergistetes are known to digest organic residues such as proteins and amino acids
Fig. 5. Phylogenetic tree of bacterial sequences retrieved from DNA in anaerobic sludges WWT and SWT as determined by the neighbor-joining method on the basis of 16S rDNA sequence. Methanobacterium subterraneum was used as an outgroup. The percentage of 1000 bootstrap samplings that supported a cluster is indicated. The scale bar indicates 0.05 nucleotide substitution per site. Number in square brackets indicates contributing GenBank accession numbers of reference nucleotide sequences. DGGE bands 2215 and 4211 were excluded from the tree due to the short sequence information obtained.

(Jumas-Bilak et al., 2009; Nelson et al., 2011; Riviere et al., 2009) and it was speculated that they play a key role in the acido-genic phase of AD (Nelson et al., 2011). A member of the genus *Anaerobaculum* of this phylum has been characterized as a fermenting bacterium with a wide range of substrates occurring in AD (Rees et al., 1997). The sequence represented by band 2201 and assigned to the phylum *Bacteroidetes* was detected in the absence of crude oil for the first two sampling days only, but at least until day 84 after crude oil addition. The ability to degrade hydrocarbons has been confirmed so far for polycyclic aromatic hydrocarbons (PAH) by one *Bacteroidetes* strain (Kwon et al., 2006).

Three other sequences affiliated to *Synergistetes* (band 2210, Fig. 3) and members of *Alpha- and Betaproteobacteria* (bands 2212 and 2215) were detected only in the degradation test bottle on day 325. Members of both *Alpha- and Betaproteobacteria* are recognized as anaerobic alkane degraders, however, using nitrate as electron acceptor (Callaghan et al., 2009). Similarly, the two unidentified bands 2211 and 2213 were restricted only to crude oil samples and were becoming more prominent over incubation time.

3.7. Community shift in sludge SWT (bio-waste treatment plant mixed with PHC-contaminated soil)

The fingerprints from the SWT sludge are shown in Fig 4 with (A) and without (B) crude oil supplementation. Intensities of bands 4202 and 4203, assigned to the phylum *Bacteroidetes*, were gradually decreasing in both sets over incubation time. This behavior is similar to band 4201, also belonging to *Bacteroidetes*, which was, in contrast, still detectable after 170 days only in the blank. Several sequences present in both parallels on all sampling
days could not be assigned to identified bacteria, but clustered with representatives of the phylum Actinobacteria (bands 4206–4208) and Synergistetes (bands 4209 and 4210), as displayed in Fig. 5. Distinct microbial communities of two anaerobic sludges according to the comparison of 16S rDNA sequences.

<table>
<thead>
<tr>
<th>Band ID (accession number)</th>
<th>Phylogenetic clusters</th>
<th>Closest match in GenBank database (accession number)</th>
<th>% Similarity</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. WWT sludge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2201 (JN698223)</td>
<td>Bacteroidetes</td>
<td>Uncultured Bacteroidetes bacterium, clone QEDR1DA08 (FJ769497.1)</td>
<td>100%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>2210</td>
<td>–</td>
<td>Uncultured bacterium, clone CB2B8 (AB274497.1)</td>
<td>73%</td>
<td>Organic solid waste degrading anaerobic digester</td>
</tr>
<tr>
<td>2211 (JN698224)</td>
<td>–</td>
<td>Uncultured bacterium, clone noFP.JH6.2 (FJ769497.1)</td>
<td>97%</td>
<td>Methanogenic sludge</td>
</tr>
<tr>
<td>2212 (JN698225)</td>
<td>Betaproteobacteria</td>
<td>Uncultured Betaproteobacteria bacterium, clone QEDNG6BE11 (CU925083.1)</td>
<td>100%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>2213 (JN698226)</td>
<td>–</td>
<td>Uncultured Unclassified bacterium, clone QEDN3AE03 (CU919652.1)</td>
<td>98%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>2214 (JN698227)</td>
<td>Synergistetes</td>
<td>Uncultured Aminanaeroba bacterium, clone QEDN9BA04 (CU926877.1)</td>
<td>96%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>2215</td>
<td>Alpha-proteobacteria</td>
<td>Uncultured Sphingobacteriales bacterium, clone GETC3PU01DDIDEM (HM985952.1)</td>
<td>94%</td>
<td>Mesophilic biogas reactor</td>
</tr>
<tr>
<td>2216</td>
<td>–</td>
<td>Uncultured bacterium, clone HAW-RM37-2-M-17558 (FPN53325.1)</td>
<td>94%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>2217 (JN698228)</td>
<td>Synergistetes</td>
<td>Uncultured Aminanaeroba bacterium, clone QEDN10CC08 (CU926332.1)</td>
<td>100%</td>
<td>Anoxygenic digester</td>
</tr>
<tr>
<td>2218</td>
<td>Synergistetes</td>
<td>Uncultured bacterium, clone synarCH03 (AY654359.1)</td>
<td>83%</td>
<td>Anaerobic digester</td>
</tr>
<tr>
<td>2219 (JN698229)</td>
<td>–</td>
<td>Uncultured bacterium, clone ATB-KM1232 (DQ390268.1)</td>
<td>98%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>B. SWT sludge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4201 (JN698230)</td>
<td>Bacteroidetes</td>
<td>Uncultured Bacteroidetes bacterium, clone QEDR1DA08 (CU922385.1)</td>
<td>100%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>4202 (JN698231)</td>
<td>Bacteroidetes</td>
<td>Uncultured Bacteroidetes bacterium, clone QEDR1DA08 (CU922385.1)</td>
<td>98%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>4203 (JN698232)</td>
<td>Bacteroidetes</td>
<td>Uncultured Bacteroidetes bacterium, clone QEDR2BH08 (CU922738.1)</td>
<td>99%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
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<td>4206 (JN698233)</td>
<td>–</td>
<td>Uncultured bacterium, clone ATB-KM1232 (DQ390268.1)</td>
<td>99%</td>
<td>Anoxygenic digester of a biogas plant</td>
</tr>
<tr>
<td>4207 (JN698234)</td>
<td>–</td>
<td>Uncultured Unclassified bacterium, clone QEDS3AH04 (CU921882.1)</td>
<td>89%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>4208 (JN698235)</td>
<td>–</td>
<td>Uncultured bacterium, clone ATB-KM1232 (DQ390268.1)</td>
<td>99%</td>
<td>Anoxygenic digester of a biogas plant</td>
</tr>
<tr>
<td>4209</td>
<td>–</td>
<td>Uncultured bacterium, clone A0-pApH-98 (EU878335.1)</td>
<td>78%</td>
<td>Anaerobic digester of a biogas plant</td>
</tr>
<tr>
<td>4210 (JN698236)</td>
<td>–</td>
<td>Uncultured bacterium, clone TUM-Mbac-MB-84-5.3-KC1 (EU812970.1)</td>
<td>86%</td>
<td>Biogas reactors fed with corn silage</td>
</tr>
<tr>
<td>4211</td>
<td>–</td>
<td>Uncultured bacterium, clone bac2 (GQ369776.1)</td>
<td>88%</td>
<td>Anaerobic bioreactor treating municipal solid waste</td>
</tr>
<tr>
<td>4212</td>
<td>Firmicutes</td>
<td>Uncultured Firmicutes bacterium, clone QEEA2BG11 (CU918759.1)</td>
<td>93%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>4213 (JN698237)</td>
<td>Firmicutes</td>
<td>Uncultured Firmicutes bacterium, clone QEDN2JG07 (CU927811.1)</td>
<td>80%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
<tr>
<td>4214 (JN698238)</td>
<td>Firmicutes</td>
<td>Uncultured Firmicutes bacterium, clone QEEA2BG11 (CU918759.1)</td>
<td>98%</td>
<td>Wastewater treatment anaerobic digester</td>
</tr>
</tbody>
</table>

a Riviere et al. (2009).

b Klocie et al. (2007).

c Weiss et al. (2009).

d Sasaki et al. (2007).

e Li et al. (2008).

f Trzcinski et al. (2010).

3.8. Adaptation of bacterial communities to exposure to petroleum hydrocarbons

Under anaerobic and especially methanogenic conditions, microbial cell division is slow (de Bok et al., 2005; Edwards and Grbic-Galic, 1994) as a result of the low energy yield of the process (Edwards and Grbic-Galic, 1994; Heider et al., 1998). For methanogenic pollutant degradation, lag times ranging from several months up to years prior to methane production have been noted in lab- and field-scale (Edwards and Grbic-Galic, 1994; van der Meer, 2006; Zengler et al., 1999). Similarly, in the present study, PHC degradation was only observed after at least 170 days of incubation in both communities. Several bands were appearing in the second half of incubation time, i.e. between days 170 and 325, while less pronounced shifts were observed in the first 170 days, in both sludges. Microbial adaptation to environmental pollutants
can proceed on a biochemical or genetic level (van der Meer, 2006). Genetic adaptation requires, in relation to generation time, a relatively long time covering multiple recombinations, mutations and horizontal gene transfers (HGTs). Biochemical adaptation to PHC includes induction and repression of existing genes resulting in enzyme synthesis and a change of cell biochemistry, and takes place on a smaller time-scale. Thus, the latter processes appear to be more likely the driving factor for the presently observed bacterial population changes.

4. Conclusion

The present results provide evidence that the treatment of PHC-containing wastes, such as crude oil contaminated soil, via anaerobic digestion is feasible from a microbial viewpoint. Over 11 months of incubation with crude oil, bacterial communities unadapted to PHC exhibited a distinct bacterial response. PHC were found to be degradable under these conditions. Several bacterial phyla previously associated with anaerobic PHC-containing environments were identified. Their role, however, in PHC degradation remains to be characterized. Provided more insight into adaptation kinetics, syntrophic interactions, also including archaeal populations, and the demand of these communities to their environment, this approach could be turned into a treatment alternative for petroleum hydrocarbon contaminated residues. The fate of hydrocarbons, i.e. the rate and extent of PHC conversion to methane, remains to be determined in order to assess the possibility for energy recovery from PHC-waste treatment. The present data, obtained using a 16S rDNA-based approach, represent first insight into these processes and consequently, further studies will focus on the metabolic interactions and activities of archaen and bacterial consortia.

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