Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Mar Pollut Bull. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Mar Pollut Bull. 2018 November ; 136: 351–364. doi:10.1016/j.marpolbul.2018.09.002.

An assessment of the microbial community in an urban fringing tidal marsh with an emphasis on petroleum hydrocarbon degradative genes.

Sinéad M. Ní Chadhain[b], **Jarett L. Miller**[a], **John P. Dustin**[a], **Jeff P. Trethewey**[a], **Stephen H. Jones**[c], and **Loren A. Launen**[a,*]

[a]Department of Biology, Keene State College, 246 Main St., Keene, NH 03435, USA

[b]Department of Biology, LSCB 217, University of South Alabama, 5871 USA Drive N., Mobile, AL 36688, USA

[c]Department of Natural Resources and the Environment, University of New Hampshire, 285 Rudman Hall, 46 College Rd., Durham, NH 03824, USA

Abstract

Small fringing marshes are ecologically important habitats often impacted by petroleum. We characterized the phylogenetic structure (16S rRNA) and petroleum hydrocarbon degrading alkane hydroxylase genes (*alkB* and *CYP 153A1*) in a sediment microbial community from a New Hampshire fringing marsh, using alkane-exposed dilution cultures to enrich for petroleum degrading bacteria. 16S rRNA and *alkB* analysis demonstrated that the initial sediment community was dominated by Betaproteobacteria (mainly *Comamonadaceae*) and Gammaproteobacteria (mainly *Pseudomonas*), while *CYP 153A1* sequences predominantly matched Rhizobiales. 24 hours of exposure to *n* -hexane, gasoline, dodecane, or dilution culture alone reduced functional and phylogenetic diversity, enriching for Gammaproteobacteria, especially *Pseudomonas*. Gammaproteobacteria continued to dominate for 10 days in the *n*-hexane and no alkane exposed samples, while dodecane and gasoline exposure selected for grampositive bacteria. The data demonstrate that small fringing marshes in New England harbor petroleumdegrading bacteria, suggesting that petroleum degradation may be an important fringing marsh ecosystem function.

Keywords

fringing marsh; *alkB*, n-alkane; sediment microbial community; New England; petroleum

---

*Correspondence: Loren A. Launen, 246 Main St. Keene NH 03431 USA, Tel: 603 358 2574, Fax: 603 358 2525 llaunen@keene.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript

Introduction

Salt marshes are frequently contaminated with petroleum through both large-scale releases, such as the Deepwater Horizon well blowout in the Gulf of Mexico in 2010 (Atlas et al., 2015; Natter et al., 2012; McGenity 2014), as well as chronic lower-level contamination from sources such as stormwater and small marine transportation related spills (McGenity 2014; Vieites et al., 2004). Microbial degradation of petroleum hydrocarbons released into marshes is the major means of removal, as evidenced by research demonstrating both the loss of petroleum, and the expansion of hydrocarbondegrading taxa and associated degradative genes (Acosta-González et al,. 2015; Atlas et al., 2015; Beazley et al., 2012; Kimes et al., 2014, Koo et al., 2015; Looper et al., 2013; Lu et al., 2012; Mahmoudi et al., 2013; Vega et al., 2009; Zhu et al., 2004). Petroleum bioremediation potential is related to the chemical composition of the polluting petroleum, the composition of the indigenous microbial communities present in the receiving environment, and factors such as marsh vegetation (AcostaGonzález et al., 2015; Atlas 1975; Atlas et al., 2015; Beazley et al., 2012). Petroleum contains hundreds of different hydrocarbons, including high quantities of *n*-alkanes (Speight 1998). *N*-alkanes, are, therefore, one of the major groups of contaminants that routinely intrude into salt marshes.

N-alkanes are some of the most biodegradable petroleum hydrocarbons, particularly under aerobic conditions where many bacteria, especially Alpha-, Beta- and Gammaproteobacteria, Actinomycetales and Firmicutes, can degrade a range of *n*-alkanes (Joye et al., 2016; Mason et al., 2012; Rojo 2009; Van Beilen & Funhoff 2007; Wang et al., 2010). Some alkane-degrading bacteria are actually obligate or near-obligate alkanotrophs, such as *Alcanivorax borkumensis* (Sabirova et al., 2006). Aerobic degradation of medium chain-length alkanes (C5-C16), is initiated by terminal carbon hydroxylation (Van Beilen & Funhoff 2007), effected by two types of alkane hydroxylases: membrane-bound non-haem diiron *alkB*type hydroxylases (Kok et al., 1989; Van Beilen et al., 1994), and soluble Class 1 cytochrome P450 CYP153A1 (*CYP 153A1*) hydroxylases (Asperger et al., 1981; Kubota et al., 2005; Maier et al., 2001). The *alkB* and *CYP 153A1* genes have been characterized in isolates and environmental DNA and in the case of *alkB* are considered indicators of enhanced microbial petroleum hydrocarbon degradation activity (Liu et al. 2015; Lu et al., 2012; Van Beilen & Funhoff 2007, and others). Some bacteria possess only *alkB*, others only *CYP 153A1*, and some have multiple alkane hydroxylase systems that work in concert (Chen et al., 2017, Liu et al., 2011; Nie et al., 2014; Schneiker et al., 2006; Van Beilen and Funhoff 2007; Wang et al., 2010a). Novel *alkB* and *CYP 153A1* novel alkane hydroxylases continue to be discovered in a variety of environments (Nie et al., 2014).

The majority of studies on petroleum degradation by salt marsh microbial communities have focussed on large scale release events due to shipping accidents or blowouts and large salt marsh meadow systems such as those found in the Gulf of Mexico area impacted by the 2010 Deepwater Horizon blowout (for example, McGenity 2014). In New Hampshire, small estuarine marshes referred to as fringing marshes comprise a significant amount of the total salt marsh habitat (Morgan et al., 2009, PREP 2013) and have important ecosystem functions and values (Morgan et al., 2009). These fringing marshes are different from large meadow marshes, possessing lower levels of organic carbon and plant density (Morgan et

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

al., 2009) and lower levels of denitrification enzyme activity (Wigand et al., 2004). Fringing marshes are the first location impacted by petroleum influx, which is considered one of the major threats to such marshes in New Hampshire (NH DES, 2004). Because the microbial communities that occupy these marshes are relatively unstudied, we cannot presently evaluate whether petroleum hydrocarbon biodegradation is an inherent fringing marsh ecosystem function (Bier et al., 2015; Bodelier 2011; Bombach et al., 2010; Graham et al., 2016).

In this study we aimed to address this knowledge gap by characterizing the catabolic and phylogenetic microbial diversity within sediment from a brackish chronically-impacted fringing marsh on the Cocheco River of the Great Bay Estuary of New Hampshire. The site has a history of chronic petroleum contamination and phytoremediation of aromatic hydrocarbons has been demonstrated through the activity of the indigenous *Spartina alterniflora* present (Watts et al. 2006). By analyzing alkane hydroxylase genes (*alkB* and *CYP 153A1*) using a clone library approach, and conducting 16S rRNA tag sequencing, we assessed both catabolic and phylogenetic diversity in the baseline microbial community, as well as the community after one and 10 days of continuous alkane exposure in dilution cultures. Dilution cultures exposed to three different sources of alkanes (gasoline, *n* -hexane and dodecane, as well as a no alkane control) were used for the purpose of enriching the numbers of alkane-degrading microbes present through selection, making them easier to detect and catalogue (Teeling and Glöckner, 2012) and facilitating a broad assessment of the petroleum hydrocarbon degrading gene pool present in the community. Given the chronic nature of petroleum input in the area, and the variable nature of redox, salinity and other chemical gradients in tidally influenced sediments, we hypothesized that the indigenous microbial community would contain detectable alkane hydroxylase genes and possess some degree of catabolic and phylogenetic diversity. Our data supported this hypothesis, revealing that the initial sediment community contained nine bacterial phyla with Beta- and Gammaproteobacteria emerging as dominant. Gammaproteobacteria continued to dominate the *n*-hexane and no alkane exposed samples for 10 days while dodecane and gasoline exposure selected for gram-positive bacteria. In total a diverse array of bacteria capable of responding to petroleum inputs into these fringing marsh systems were detected suggesting that petroleum hydrocarbon degradation is an ecosystem function conferred by the marsh microbial community.

Materials and Methods

Sediment sampling and dilution cultures

Sediment samples were collected from a tidal marsh vegetated with *Spartina alterniflora*, located along the Cocheco River in Dover, NH (43°11’51.36”N 70°52’02.81”W) on 11 May 2011 (after spring plant emergence) at low tide. The Cocheco River is listed as impaired (NHDES 2015) due to the chronic input of petroleum hydrocarbon contamination from shipping and other sources (Magnusson et al., 2012) as well as historical and current point and non-point sources including waste water treatment plants, landfill leachate (such as the Superfund listed Tolend Road landfill in Dover NH: EPA) and urban stormwater. Because the level of impervious surfaces is increasing (PREP 2013) stormwater-carried

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

contaminants, such as petroleum hydrocarbons (Brown et al., 2006; Makepeace et al., 1995), routinely enter the river and its tidal marsh sediment. Several sediment samples were collected from the top 15 cm of a 3 m x 3 m area containing vegetated and un-vegetated areas. These were composited into a sterile 1 gallon container. Large plant material, rocks or other visible detritus was removed by hand and the sample was homogenized by passing through a Food Mill (RSVP International Inc, Seattle, Washington) using two different filtering disks with pore size 0.5 cm and 0.2 cm.

The composited sediment sample was used to establish four dilution cultures; three receiving different sources of n-alkanes (gasoline (G), *n*-hexane (H) or dodecane (D)), and one that was unamended (the "no alkane" control flask (NA)). Each dilution culture contained 1 g (wet weight) of the composited sediment and 50 mL modified Minimal Salts Broth (Launen et al., 2008) in 250 mL Erlenmeyer flasks. All cultures were maintained at room temperature and shaken at 100 rpm for 10 days. Gasoline (G) and *n*-hexane (H), which are highly volatile, were administered in the vapor phase by maintaining a constant supply of 1.5 mL of each in suspended vials within the respective culture flask throughout the 10 day experiment. Dodecane (D), which has very low volatility, was administered neat by adding 5 μL of dodecane initially and then again every 48 hours throughout the 10 day experiment. The aim of this dosage regimen was to provide a continuous alkane supply to the microbial communities present throughout the 10 days of the experiment. Samples (one 4 mL sample per flask) were collected as follows: the baseline sediment microbial community composition was catalogued by sampling the no alkane (control) dilution culture immediately after establishing it and shaking it briefly. This was the T0 sample that was considered representative of the *in situ* microbial community present in the marsh (baseline). Each of the four dilution cultures (the NA, G, H and D cultures) were subsequently sampled (one sample per flask) at day 1 (T1) and 10 (T10) for genomic DNA extraction and subsequent processing (described below). The NA dilution culture was included as a control for changes in the microbial community due to dilution culture alone.

DNA extractions and clone library preparation and sequencing

Genomic DNA was extracted from 4 mL samples collected from dilution cultures (as above). Extractions were done using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). PCR was conducted using primers that targeted the *alkB* alkane hydroxylase (Kloos et al.,2006; Van Beilen et al.,2006; Wang et al.,2010b). The forward primer of Kloos et al., was modified by addition of an R on the 3' end. PCR reactions were conducted using 1μM of forward and reverse primers, 2X Green GoTaq Reaction Buffer (Promega, Madison, WI), 1 μL template DNA (ca. 10 – 50 ng) and nuclease-free water (to 20 μL). The amplification cycle consisted of an initial denaturing step of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and a final elongation of 72°C for 5.5 minutes.

Hydroxylase gene fragments (both *alkB* and *CYP 153A1*) were cloned into pCR4-TOPO vectors (Life Technologies, Carlsbad, CA). Plasmid DNA was isolated using the PureYield Plasmid Miniprep System (Promega). Sequencing was conducted by the University of

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Washington High Throughput Genomics Center using either the M13 forward or reverse primers provided by the sequencing facility.

Phylogenetic analysis of clone library sequences

Primer sequences were removed from nucleotide sequences using Geneious v. 7.1.5 (created by Biomatters). Sequences were aligned with a ClustalW alignment (in Geneious) and Phylip v. 3.695 (Felsenstein 2005). An alignment-associated DNA distance matrix was input into the program MOTHUR v. 1.33.1 (Schloss & Handelsman 2009), that was used to cluster sequences into operational taxonomic units (OTUs) based on the nearest neighbor algorithm and a 95% (nucleotide) sequence identity.

MOTHUR was also used to construct rarefaction curves for each library, and to calculate estimates of nonparametric richness (Chao1 and Shannon-Weaver diversity indices) (Chao 1984; Chao and Lee 1992; Pielou 1977). Phylogenetic trees were constructed from the ClustalW alignment of the derived amino acid sequences by neighbor-joining analysis with 1000 bootstrap replicates using MEGA 5.0 (Tamura et al., 2011).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this study were deposited in the GenBank database with the following accession numbers KT280353 to KT280397 (selected representatives of each *alkB* OTU) and KT305744 to KT305774 (selected representatives of each *CYP 153A1* OTU).

Quantification of relative *alkB*, *CYP 153A1* and *16S* rRNA gene abundance in dilution cultures

The dominant *alkB* and *CYP 153A1* OTUs from the clone libraries were further analyzed using real-time PCR. QPCR primers (Table S1) were designed from the nucleotide sequences of clones from within the selected OTUs using Primer Express (V3.0.1, Applied Biosystems). The abundance of selected *alkB* and *CYP 153A1* OTU sequences was determined relative to the abundance of bacterial 16S rRNA genes. Standard curves were generated using two representative plasmid clones from each OTU. Plasmid DNA was linearized with the *NotI* restriction enzyme and quantified using a Nanodrop 2000 (Thermo Orion). Standard curves for bacterial 16S rRNA genes were generated using *Escherichia coli* JM109 genomic DNA. QPCR reactions were performed in 96-well Fast Optical MicroAmp reaction plates (Life Technologies) where each reaction had a total volume of 12.5 μl: 1 μl of environmental sample or plasmid DNA solution, 6.25 μl SYBR Green PCR Master Mix (Applied Biosystems), 4.75 μl water and 0.25 μl of each primer. Thermal cycling and quantification were performed using a StepOne Plus RealTime PCR System (Applied Biosystems) instrument programmed as recommended by the manufacturer (95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at the primerspecific annealing temperature (Table 3) for 1 min, and polymerase extension at 72°C for 1 min. The final primer concentration was 0.2 μM. Amplification specificity was achieved for each primer set, as determined by the presence of a single peak in each post-amplification dissociation curve.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Bacterial community sequencing

PCR amplification of the 16S rRNA hypervariable region V6 was performed with a pool of degenerate forward and reverse primers targeting bacteria as described by Huber at al., (2007). The 5′-ends of the forward primers were fused with the Ion Torrent A-Adaptor plus a MID key sequence (barcode), while the reverse primers were fused with the truncated P1-adapter sequence (trP1). The primers were diluted in molecular grade laboratory water and pooled in equimolar concentrations for each barcode set. Template from each treatment was amplified with a different barcode.

For amplicon library preparation purified genomic DNA from each treatment was amplified in 20 μL reactions as follows: 1 μL of DNA (10–50 μg/mL concentration), 10 μL 1X Phusion PCR master mix (Thermo, Pittsburgh, PA), and 2 μL of 0.5 μM forward and reverse primers. The PCR conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 15 s, 58°C for 15 s, 68°C for 10 s, and a final elongation step of 68°C for 30 s. Triplicate amplifications were performed for each treatment. PCR products were run on 2% 1X TBE gels. Amplicons of the correct size were excised, pooled, and purified with the Promega SV Gel and PCR Clean-Up kit (Promega, Madison, WI). Amplicon concentration was estimated with the Qubit 2.0 instrument using the Qubit dsDNA HS assay (Life Technologies). The bar coded amplicons were combined and diluted to 2.8×10^{8} DNA/μL molecules per micro liter prior to template preparation.

Sequencing template was prepared using the Ion OneTouch 200 Template Kit with the corresponding protocol (Pub. Part no. 4474396 Rev. B, 20 Feb 2012). Sequencing of the amplicon libraries were then carried out using the Ion Torrent Personal Genome Machine (PGM) system using the Ion Sequencing 200 kit (from Life Technologies) and the corresponding protocol (Pub Part Number 4474596 Rev. C, 10 Oct 2012) on Ion Torrent 314 chips.

Bacterial community sequence analysis

Raw sequencing reads were processed using the Quantitative Insight Into Microbial Ecology (QIIME) open source software package and the Virtual Box QIIME package (Caporaso et al., 2010a). Raw sequences were filtered and screened for different quality criteria. Low quality reads were removed as follows: i) all reads <70 b.p. were removed; ii) all reads with quality scores of lower than 20 were removed; iii) no ambiguous reads were allowed; and iv) reads with homopolymers of 4 b.p. or less were removed to reduce sequencing error. Chimeras rarely form in the V6 regions of the sequence due to its small size (approximately 70– 200 b.p. sequence lengths) so the data were not screened for chimeras.

Subsequently, de novo OTUs were clustered at a 97% identity threshold and aligned to the Greengenes database (DeSantis et al 2006) using PyNAST (Caporaso et al., 2010b). Sequences were processed using the *pick_open_reference_otus.py* workflow with the default values and uclust (Edgar, 2010). Taxonomy was assigned using the August 2013 release of Greengenes (DeSantis et al., 2006) and the naïve Bayesian RDP classifier (Wang et al., 2007). Phylogenetic trees were constructed using FastTree 2 (Price et al. 2010). Alpha diversity was calculated using the QIIME pipeline. The default metrics for the QIIME

Author Manuscript

pipeline alpha diversity measurement were edited to include the Shannon diversity index and phylogenetic distance, in addition to Chao1 and observed species. The minimum number of sequences used for depth of coverage in alpha rarefaction was set to 5000 for even sampling. Beta diversity metrics were also generated using QIIME. 5000 sequences were subsampled from each dilution sample for calculation of beta diversity using both weighted and unweighted unifrac (Lozupone & Knight 2005) analyses. Principal Coordinate Analysis (PCoA) (Vazquez-Baeza et al., 2013) and hierarchical clustering (UPGMA) (Caporaso et al., 2010a) were utilized to visualize the data structure and differences between the samples. Jackknife replicates (100 replicates) were used to estimate the uncertainty in both the PCoA and hierarchical clustering plots.

Results

Analysis of alkane hydroxylase clone libraries

AlkB* clone library—AlkB* alkane hydroxylase gene fragment clone libraries were prepared from genomic DNA extracted from alkane-exposed and no alkane control dilution cultures over 10 days. The distribution of OTUs, their diversity, and matches to known sequences as determined by Blastx are shown in Table 1 and 2, and Fig. 1A and 2. Dilution culture alone reduced diversity of *alkB* gene fragments according to the Shannon-Weaver diversity index and Chao1 estimates (Table 1) in the first day of dilution culture, however, both measures increased by 10 days (T10) to levels greater than the initial T0 sediment indicating that *alkB* diversity rebounded in the dilution culture. In contrast, all alkane treatments reduced *alkB* gene fragment diversity to levels less than those found in the no alkane control sample after 1 and 10 days of continuous alkane exposure.

The taxonomic assignment of *alkB* sequences recovered from each sample library differed according to treatment (Table 2, Fig. 1A). The initial sediment (the T0 sample), yielded 25 *alkB* clone library sequences which contained 14 different OTUs, the majority of which were an 81% BlastX match to *alkB* from *Pseudomonas aeruginosa* (OTU2) or an uncultured *Comomodaceae* sequence (OTU11) (see Fig. 1A). After one day of dilution culture, the no alkane control sample (T1 NA) yielded 14 clone library sequences, containing 9 OTUs, most of which matched OTU2 (*P. aeruginosa*). By 10 days of dilution culture alone (T10 NA) 26 *alkB* clone sequences were recovered, containing 15 OTUs, with the majority matching OTU8 (uncultured/*Alcanivorax* sequences as best match) and OTU9 (*Kordiimonas gwangyangensis*).

AlkB libraries prepared from *n*-hexane and gasoline amended samples yielded fewer OTUs overall and possessed reduced diversity measures relative to the no alkane control libraries (Table 1). One day of exposure to both *n*-hexane (T1 H) and gasoline (T1 G) resulted in a selection for the *P. veronii*-like OTU1 (Fig. 1A, 14/29 and 25/31 of the clone sequences from these libraries respectively). This selection for *Pseudomonas*-type *alkB* sequences persisted into the 10 day samples where 18/24 (T10 H) and 16/21 (T10 G) were also sequences belonging to OTU1 (*P. veronii*). Dodecane appeared to impact the *alkB* diversity differently than exposure to *n*-hexane or gasoline. While the one day dodecane-exposed sample was dominated by OTUs that match best to *Pseudomonas* spp. (Table 1 and Fig. 2A), by 10 days of dodecane exposure *alkB* sequences were predominantly OTU4 (15/19 sequences

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

recovered), which is a 99% BlastX match to an *alkB* from *Rhodococcus* sp. RP-11 (Table 2, Fig. 1A). OTU4 (*Rhodococcus* sp. RP-11) was the major gram-positive type *alkB* identified in any *alkB* library prepared in this study.

Differing profiles of *alkB* clone sequences were observed in each type of dilution culture. *N*-hexane and gasoline treatment selected for *alkB*s of *Pseudomonas*-type, dodecane treatment selected initially for *Pseudomonas*-type sequences with a switch to *Rhodococcus*-type *alkB* sequences by day 10, and the no alkane control dilution culture selected for an initial increase in *Pseudomonas aeruginosa*-type *alkB* sequences, with a switch to uncultured/ *Alcanivorax* and *Kordiimonas gwangyangensis* type *alkB* sequences by day 10.

Rarefaction curves (Fig. S1A) constructed for each *alkB* library indicated that all libraries except the T0 and the 1- and 10-day no alkane control libraries were adequately sampled (ie. plateaus were observed). This suggests that exposure to alkanes, as well as dilution culture alone, simplified the *alkB*-gene pool through selection for particular microbial community members.

CYP 153A1* clone library analysis—CYP 153A1* clone libraries were constructed from amplicons from the initial sediment sample (T0), the no alkane exposed control one and ten day samples (T1 NA and T10 NA), and the 1- and 10-day dodecane exposed samples (T1 D and T10 D), see Fig. 1B, 3 and Table 1. The other samples did not yield sufficient *CYP 153A1* PCR product for library construction. Furthermore, the T1 NA and the T1 and T10 D libraries contained very few sequences (Table 1). The T0 initial sediment sample contained sequences that assigned to 13 OTUs and had the highest Chao1 and Shannon Weaver scores (see Table 1). These sequences matched by BlastX to *CYP 153A1* from the alpha proteobacterial order Rhizobiales, and genera *Sphingopyxis*, and *Parvibaculum,* as well as several uncultured bacteria that group with the Alphaproteobacteria (Fig. 3). The T1 NA library yielded only three sequences, each belonging to different OTUs; one Rhizobiales-like sequence (OTU6), a *Rhodococcus* (OTU27) and *Parvibaculum hydrocarboniclasticum* (OTU31). T10 NA contained predominantly *P. hydrocarboniclasticum* (OTU3) and Rhizobiales (OTU2). The only sequence recovered from the one-day dodecane (T1 D) exposed sample environment most closely matched a *CYP 153A1* from an uncultured Rhizobiales (OTU16) (Fig. 1B, Table 2). By 10 days of dodecane exposure most of the *CYP 153A1* sequences matched either *CYP 153A1* sequences from *Rhodococcus fasciens* (OTU1) or *Rhodococcus erythropolis* (OTU7).

Analysis of rarefaction curves (Fig. S1B) constructed for each *CYP 153A1* library indicated that only the 10-day exposure libraries (dodecane and no alkane) reached a plateau and thus were adequately sampled. Overall, the limited number of sequences recovered limits our ability to analyze *CYP 153A1* alkane hydroxylases from the microbial community studied. It is interesting that the T10 D sample yielded both *Rhodococcus*-type *CYP 153A1* and *alkB* alkane hydroxylase sequences.

Quantification of relative *alkB* and *CYP153A1* gene abundance in dilution cultures

QPCR was used to assess the relative abundance of the dominant alkane hydroxylase OTUs from the dilution cultures. Primers were designed to target *alkB* OTUs 1–5 and *CYP 153A1*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

OTUs 1, 4 and 7 (Table 2) (primers are in Supplementary Table 1, results in Table 3). *AlkB* OTU5 (*Pseudomonas lini*) was the only *alkB* OTU detected in the T0 sample DNA. This OTU was detected in all samples except for the 10 day no alkane control sample, suggesting that it may not be specifically induced by alkanes. The T1 H sample contained elevated OTU2 levels, a sequence which matches an alkane hydroxylase from *Pseudomonas aeruginosa* (Table 2). OTUs 1 and 3 (also matches to *Pseudomonas*-derived alkane hydroxylases) were also detected in this sample, although at lower levels. Ten days of hexane exposure (T10 H) resulted in the decline of OTUs 2 (*Pseudomonas aeruginosa*) and 3 (*Pseudomonas aeruginosa*), and an approximately 7-fold increase in OTU1 (*Pseudomonas veronii*). In the gasoline-exposed samples, OTUs 1 (*Pseudomonas veronii*), 3 (*Pseudomonas aeruginosa*) and 5 (*Pseudomonas lini*) were found at the same levels in the 1- day and 10-day samples. OTU2 (*Pseudomonas aeruginosa*) was not detected in the 1- day sample but was present at relatively high abundance in the 10- day gasoline-exposed sample (T10 G). The 1-day dodecane exposed sample contained detectable *alkB* OTUs 1, 2, 3 and 5 and no detectable OTU4 (*Rhodococcus* sp. RP-11). By day 10, however, OTU4 (*Rhodococcus* sp. RP-11) dominated. Overall, the *alkB* qPCR analysis supported the clone library analysis, demonstrating a selection for *Pseudomonas*-type *alkBs* in response to *n*-hexane and gasoline treatment, and for *Rhodococcus*-type *alkB*s in response to dodecane treatment.

The *CYP 153A1* qPCR analysis was largely unsuccessful, with no detection of any of the selected OTUs other than the OTUs 1 (*Rhodococcus fascians*), 4 (uncultured Rhizobiales-like) and 7 (*Rhodococcus erythropolis*) in the 10- day dodecane-exposed sample. This may suggest *CYP 153A1* type alkane hydroxylases were not important in alkane degradation in this system, or may be due to limitations in our primer design.

16S rRNA sequence analysis

The composition of the initial sediment microbial community (T0) as well as the community present in dilution cultures exposed to different alkanes was examined by tag sequencing of the V6 region of the 16S rRNA using an Ion Torrent PGM. A total of 321,310 reads were obtained, of which 281,782 passed quality filtering and were mapped to individual barcodes using the QIIME pipeline. The number of sequences mapped to each sample ranged from a high of 68,610 in the initial sediment (T0) sample to a low of 92 sequences in the day 10 no alkane control dilution culture sample (T10 NA) (Table 4). The processed data were then used to calculate alpha (Shannon, Chao1, phylogenetic distances and PCoA analyses; Table 4 and Fig. 6) and beta (Unifrac analysis; Fig. 5) diversity metrics at a sampling depth of 5000 with T10 NA excluded due to the low read number (Table 4).

Between 93.6% and 99.5% of the processed sequences could be classified below the bacterial root using the QIIME pipeline with the majority of sequences classified to the family or genus level. OTUs were divided into 13 groups for ease of analysis (Fig. 4), with each Group assigned to a genus, family or Class, as could be achieved by our analysis. Group I comprised the sequences that could not be classified and accounted for between 0.5 and 6.4% of all sequences obtained. Group XIII represents all rare OTUs (ie. each sequence type is 1% of the sequences from a given sample) which were pooled and together form

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Group XIII. The taxonomic annotation for the remaining groups is included in the Fig. 4 legend.

The initial sediment bacterial community (T0) yielded 98,610 processed sequences which were assigned to 2,132 OTUs (Table 4). This sample was the most diverse sample in this study by all measures other than the Chao1 score (Table 4). 6.4% of these sequences could not be taxonomically assigned (Group I). The number of sequences assigned to Group XIII (rare, pooled sequences, Fig. 4) was 37.8%. Of the remaining sequences, the dominant phyla were the Betaproteobacteria (19.6%), Gammaproteobacteria (11.8%), *Bacteroidetes* (7.3%), Deltaproteobacteria (5.6%), *Firmicutes* (4.7%) and Alphaproteobacteria (3.5%). The most abundant genus present was *Pseudomonas* (3.2% - Group XII). The initial sediment bacterial community (T0) sample contained some sequences not found in any other sample, such as *Geobacter* (2.8%), *Clostridium* (2.8%), *Flavobacterium* (2.4%) and *Burkholderiales* (10%). The grampositive sequences represented 4.8% of the total (4.7% *Firmicutes* and 0.1% *Actinobacteria*).

Dilution alone (the no alkane dilution culture), reduced diversity and altered the community composition (Table 4). The T1 NA sample was less diverse than the T0 sample by all measures calculated (Table 4), containing a community dominated by Gammaproteobacteria (84.7%) mainly *Acinetobacter* (Group XI: 43.5%) and *Pseudomonas* (Group XII: 26.9%). The T10 NA sample yielded only 92 reads and thus could not be assessed for diversity metrics. Of the 92 reads 22.8% were Gammaproteobacteria (the dominant taxon) of which 8.7% were Group IX *Marinobacter*.

One day of exposure to *n*-hexane and gasoline reduced the diversity of the communities sampled beyond the loss of diversity seen in one day of dilution alone (the no alkane control, T1 NA; Table 4).

Interestingly, this was not the case with dodecane exposure; after one day of exposure the dodecane exposed dilution culture sample (T1 D) contained a microbial community that was more diverse than the initial sediment microbial community (T0) by all measures assessed. Comparing the diversity measures in all three types of alkane exposed dilution cultures (T10 D, T10 H and T10 G; Table 4) after 10 days indicates that diversity continued to decline in the *n*-hexane and gasoline exposed cultures, and to a lesser degree in the dodecane exposed culture. Hierarchical clustering (Fig. 5) from unweighted and weighted unifrac analyses revealed the dodecane-exposed samples from both 1 and 10-day samples, clustered with the initial T0 sample, separate from all other samples. PCoA analysis (Fig. 6) revealed that the similarities between the different hydrocarbon amendments and the no alkane control dilution culture were driven mainly by the Gammaproteobacteria.

One day of exposure to *n*-hexane and gasoline shifted the community to predominantly Gammaproteobacteria (94.1 and 96.0% respectively). This gamma proteobacterial dominance persisted to 10 days in the *n*-hexane exposed culture, but in the gasoline-exposed culture the proportion of Gammaproteobacteria declined by 10 days and an increase in Alphaproteobacteria, specifically the family *Acetobacteraceae* (Group VII: 22.5%) occurred. The proportion of gram-positive bacteria increased in the T10 G sample to 29.7%,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

predominantly Firmicutes from the genera *Bacillus*, *Lysinibacillus* and *Rumelibacillus*. As observed in the analysis of *alkB* clone sequences, dodecane exposure caused a transient shift towards Gammaproteobacteria (56.2%) after one day of exposure, but by 10 days of continued dodecane exposure the Gammaproteobacteria declined (to 3.1% of sequences), and an upsurge in gram-positive bacteria (to 59.0%) occurred. The gram-positive bacteria were mainly from the class *Actinobacteria*, chiefly *Mycobacterium* (Group II: 32.4%) and *Rhodococcus* (Group III: 26.2%). Overall, the T10 D sample contained the greatest proportion of gram-positive sequences observed in any sample (The T0 sample contained 4.8%, all T1 samples contained less than one percent, T10 H contained 3.9%, T10 G 29.7% and T10 D 59.0%). In summary, the analysis of the microbial community agrees with the *alkB* clone library analysis, indicating that Gammaproteobacteria are important members of the microbial communities within the initial marsh sediment that are able to respond quickly to both dilution and alkane exposure.

Discussion

Small brackish estuarine tidal marshes, referred to as fringing marshes, are an important coastal habitat in New Hampshire (Morgan et al., 2009 and references therein; PREP 2013), thought to provide essential ecosystem services similar to larger meadow marshes. These fringing marshes are under pressure from sea level rise and exposure to pollutants such as petroleum (Morgan et al., 2009; NHDES 2004; PREP 2013; Stralberg et al., 2011). Unlike larger meadow marshes, relatively little is known about the ability of fringing marsh microbial communities to degrade petroleum hydrocarbons, knowledge that would help us better predict marsh resilience. In this study we catalogued the microbial community in a fringing marsh from the Cocheco River of the Great Bay of New Hampshire with an emphasis on the alkane hydroxylase gene pool. To our knowledge, this is the first inventory of microbial diversity and alkane hydroxylase genetic potential conducted in an urban fringing marsh system. The study site is typical of fringing marshes in New Hampshire with regards to vegetation (it is sparsely vegetated with *Spartina alterniflora*) and petroleum hydrocarbon influx. Phytoremediation of polyaromatic hydrocarbons has previously been documented at this site (Watts et al., 2006) which has a history of petroleum hydrocarbon contamination.

We used a dilution culture approach, exposing the initial microbial community to three different sources of alkanes (gasoline, *n*-hexane and dodecane, as well as a no alkane control culture) in order to catalogue the initial sediment community, as well as the community present after applying alkanes as a selection pressure. This approach allowed us to broadly assess the microbial diversity present and the genetic potential for alkane degradation initially, and in a simplified community (National Research Council, 2007; Teeling and Glöckner, 2012). This approach does not include replication of dilution culture treatments however, limiting our ability to compare the effect of treatment types (alkane) over time (which was not one of our goals) (Lennon 2011).

By focusing on alkane hydroxylase genes (*alkB* and *CYP 153A1*) using a clone library approach, the functional diversity was assessed in the initial sediment, and dilution cultures treated for 10 days. The initial community was already enriched in *Pseudomonas*-type *alkB*

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

sequences which increased in relative abundance by one day's exposure to dilution alone, and all three sources of alkanes tested. The initial dominance and rapid expansion of Gammaproteobacteria, especially *Pseudomonas* spp. has been shown in a variety of environments including meadow salt marshes (Acosta-Gonzáles et al., 2015; Atlas et al., 2015; Beazley et al., 2012; Kasai et al., 2001; Koo et al., 2015; Kostka et al., 2011; Lamendella et al., 2014; Liu & Liu 2013, Looper et al., 2013). By 10 days of further culture we observed a differential response according to treatment condition, with gasoline and *n*-hexane further enriching the fraction of *Pseudomonas*-type *alkBs* and reducing the overall diversity of *alkBs* recovered to a greater extent than that seen in the no alkane control or the dodecane exposed cultures. In contrast, dodecane exposure enriched for *Rhodococcus*-like *alkB* sequences and resulted in a 10- day community with slightly more diverse *alkBs* than seen in the no alkane control.

Interestingly we were only able to amplify *CYP 153A1* -type genes from the no alkane control and dodecane dilution cultures and failed to detect *CYP 153A1* genes in most samples using QPCR (Table 1, Table S1). It is possible that the primers we used to recover *CYP 153A1* type alkane hydroxylase genes were not fully effective. However, our results may also suggest that the number of *alkB* type alkane hydroxylases in the fringing tidal marsh sediment exceeds the number of *CYP 153A1* type alkane hydroxylases. Greater abundance of *alkB* type genes relative to *CYP 153A1* genes was found by Nie et al. (2014) who analyzed 3,979 microbial genomes and 137 metagenomes from a variety of environments, suggesting *alkB* may be more important than *CYP 153A1* hydroxylases in a variety of habitats. This question may be answered by future interrogation of a metagenomics dataset obtained from the fringing marsh focused on in this study (data available at the NCBI SRA, SAMN09266700).

Analysis of the 16S rRNA V6 region to assess the phylogenetic diversity and taxonomic composition of the whole community generally supported the findings of the clone library functional analysis. The initial sediment community was highly diverse, with the dominant taxa present (3.2%) matching to *Pseudomonas* spp. All dilution cultures, including the no alkane control, exhibited a decrease in diversity, richness and phylogenetic distance (PD) after one day of dilution culture (Fig. 4, Table 4) which is expected given that dilution alone reduces diversity (Franklin et al., 2001, Hewson et al., 2003, Schaffer et al., 2000, Yan et al., 2015). Comparing the diversity values obtained after one and 10 days of dilution culture (excepting the T10 NA control sample which had to be excluded due to low read number), indicated that exposure to gasoline and *n*-hexane may reduce diversity to a greater degree than seen in the dodecane treatment or the no alkane control treatment. This agrees with our functional gene analysis. Our experimental design, and our inability to assess the 10 day NA control sample due to low read number, limit the conclusions we can make from this observation. However, other studies document the negative impact of highly concentrated petroleum hydrocarbons on microbial diversity in meadow-type salt marsh sediment and other habitats (Acosta-González et al., 2015; Atlas et al., 2015; Beazley et al., 2012; Guibert et al., 2012; Mason et al., 2012; Pérez-de-Mora et al., 2011; Saul et al., 2005). Typically, a rebound in diversity occurs once petroleum hydrocarbon concentrations decline (through degradation: Atlas et al., 2015; Koo et al., 2015; Paisse et al., 2010). Further study is needed to better assess both loss of diversity and the potential for rebound of diversity in fringing

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

marsh sediment habitats to determine whether fringing marshes are as resilient as larger meadow marshes (Acosta-González et al., 2015; Atlas et al., 2015 and others).

The taxonomic composition of the entire microbial community varied by treatment, generally supporting the taxonomic assignment of *alkB* genes recovered. Gammaproteobacteria, particularly the rapidly responding *Pseudomonads*, dominated in the initial sediment and were enriched in all one day dilution cultures (alkane-amended or no alkane control) (Fig. 4), as well as in the 10 day gasoline and *n*-hexane exposed cultures. Gammaproteobacteria are known to respond rapidly to dilution alone (Yan et al., 2015), as well as being important early responders in petroleum-contaminated sites including larger salt marshes (references above).

Interestingly, by 10 days of exposure to dodecane and gasoline, there was an increased proportion of gram-positive bacteria, with the T10 D sample containing 59.0% gram-positive sequences mainly from the genera *Rhodococcus* (26.2%) and *Mycobacterium* (32.4%), while the T10 G sample was dominated by Firmicutes (29.7%), mainly from the genera *Bacillus*, *Lysinibacillus* and *Rumelibacillus*. *Rhodococcus* species are known alkane degraders (Hassanshahian et al., 2010, Larkin et al., 2005, Liu & Liu, 2013, Sekine et al., 2006, Van Hamme & Ward 2001, Whyte et al., 1998 & 2002, Xu et al., 2007) documented in petroleum-impacted salt marshes (Acosta-González et al., 2015; Mahmoudi et al., 2013, Liu & Liu 2013), as are *Mycobacterium* (Atlas et al., 2015 and others). The role of gram-positive bacteria in petroleum degradation has been reported in larger salt marshes and other systems (Acosta-González et al., 2015; Atlas et al., 2015; Koo et al., 2015; Launen et al., 2008; Looper et al., 2012; Liu & Liu 2013; Mahmoudi et al., 2013) and there is evidence that gram-positive bacteria may be important in the secondary response to petroleum degradation, increasing in abundance more slowly than the rapidly responding Gammaproteobacteria (Koo et al., 2015). Our results suggest that the role of gram-positive bacteria in responding to alkanes in fringing marshes may be similar.

While limited by our experimental design, we observed selection of certain microbial community members over others when challenged with different types of n-alkanes. This may reflect differences in alkane-metabolizing enzymes, or physiological differences that affect other aspects of alkane metabolism between microbes (Grund et al., 1975; Larkin et al., 2005; Smits et al., 2002; Van Beilen et al., 1994; Van Beilen et al., 2005; Wang & Shao 2013; Wentzel et al., 2007), alkane toxicity (Ramos et al., 2002; Vermuë et al., 1993; Walker & Colwell 1975), alkane uptake from the environment (Bouchez-Naïtali et al., 1999; Rosenberg & Rosenberg 1981; Van Hamme & Ward 2001), biosurfactants (Desai & Banat 1997; Ivshina et al., 1998; Pornsunthorntawee et al.,2008, Pi et al.,2017) or relative growth rates (VieiraSilva & Rocha 2010). Conclusively establishing whether specific alkanes reproducibly select for specific community members as our observations suggest here requires further study, potentially using a combination of metagenomics and culture-based approaches.

Overall, we determined that the fringing marsh studied contained a diverse microbial community that included nine different phyla, with an enrichment of *Pseudomonas*-type alkane degrading bacteria able to expand rapidly when exposed to environmental change

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(dilution and alkane exposure), as has been observed in larger meadow-type salt marshes elsewhere (Acosta-González et al., 2015; Beazley et al., 2012; Mason et al., 2012). The importance of gram-positive bacteria, especially *Rhodococcus,* in a slower response to dodecane exposure was also observed, and agrees with findings in meadow marshes and other environments (Acosta-González et al., 2015; Atlas et al., 2015; Mahmoudi et al 2013, Liu & Liu 2013). Overall, our data suggest that the fringing marsh microbial community is capable of responding to perturbation by alkanes. Given the importance of fringing marshes in the New England region, and the threat of petroleum influx into these marshes, further research into the biodegradative capacity of the microbial communities present is merited. In particular, studies that specifically measure alkane (and total petroleum hydrocarbon) degradation directly should be conducted to ensure that the genetic response reported here is indeed coupled to alkane degradation. It would also be worthwhile to directly evaluate the ability of these communities to degrade whole-oil mixtures, such as home heating oil, which is regularly transported in the Great Bay Estuary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Ms. Katie Featherston, Ms. Marianne O'Brien, Ms. Audrey Arsenault, Dr. Penny Micele and Dr. Gordon Leversee for support in the administration of this work.

Funding

This work was supported by the National Institutes of Health Institutional Development Award Program (National Institute of General Medical Sciences), [Grant No. P20GM103506].

References

Acosta-González A, Martirani-Von Abercron SM, Rosselló-Móra R, Wittich RM, and Marqués S (2015). The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the Prestige oil spill. Environ. Sci. Pollut. Res. Int 22, 15200–15214. doi: 10.1007/s11356-015-4458-y [PubMed: 25869434]

Asperger O, Naumann A & Kleber HP (1981) Occurrence of cytochrome P450 in Acinetobacter strains after growth on n-hexadecane. FEMS Microbiol Lett 11: 309–12. doi: 10.1111/j.15746968.1981.tb06986.x

Atlas RM (1975) The effects of temperature and crude oil composition on petroleum bioremediation. Appl. Microbiol 33 (3): 396–403

Atlas RM, Stoeckel DM, Faith SA, Minard-Smith A, Thorn JR & Benotti MJ (2015) Oil biodegradation and oil-degrading microbial populations in marsh sediments impacted by oil from t he deepwater horizon well blowout. Environ. Sci. Technol 49 (14): 8356–8366. doi: 10.1021/acs.est.5b00413 [PubMed: 26091189]

Beazley MJ, Martinez RJ, Rajan S et al.,(2012) Microbial community analysis of a coastal salt marsh affected by the Deepwater Horizon oil spill. PLoS ONE 7: e41305. doi: 10.1371/journal.pone.0041305. Epub 2012 Jul 18. [PubMed: 22815990]

Bier RL, Bernhardt ES, Boot CM, Graham EB, Hall EK, Lennon JT, Nemergut DR, Osborne BB, RuizGonzalez C, Schimel JP, Waldrop MP & Wallenstein MD (2015) Linking microbial community structure and microbial processes: an empirical and conceptual overview. FEMS Microbiol Ecol 91 (10): fiv113. doi: 10.1093/femsec/fiv113

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Bodelier PLE (2011) Toward understanding, managing, and protecting microbial ecosystems. Front Microbiol 2: Article 80. doi: 10.3389/fmicb.2011.00080

Bombach P, Richnow HH, Kästner M & Fischer A (2010) Current approaches for the assessment of in situ biodegradation. Appl Microbiol Biotechnol 86:839–852 doi 10.1007/s00253-010-2461-2 [PubMed: 20174793]

Bouchez-Naïtali M, Rakatozafy H, Marchal R, Leveau JY& Vandecasteele JP (1999) Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. J Appl Microbiol 86: 421–8. doi: 10.1046/j.1365-2672.1999.00678.x [PubMed: 10196747]

Brown DG, Gupta L, Kim TH, Moo-Young HK & Coleman AJ (2006) Comparative assessment of coal tars obtained from 10 former manufactured gas plant sites in the eastern United States. Chemosphere 65: 1562–1569. doi: 10.1016/j.chemosphere.2006.03.068 [PubMed: 16698063]

Caporaso J, Kuczynski J, Stombaugh J et al.,(2010a) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335–336. doi:10.1038/nmeth.f.303 [PubMed: 20383131]

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL & Knight R (2010b) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26: 266–7. doi: 10.1093/bioinformatics/btp636. Epub 2009 Nov 13. [PubMed: 19914921]

Chao A (1984) Nonparametric estimation of the number of classes in a population. Scand J Stat 11: 265–70. doi: 10.2307/4615964

Chao A & Lee S-M (1992) Estimating the number of classes via sample coverage. J Am Stat Assoc 87: 210–217. doi 10.1080/01621459.1992.10475194

Chen W, Li J, Sun X, Min J & Hu X (2017) High efficiency degradation of alkanes and crude oil by a salt-tolerant bacterium Dietzia species CN-3. Int Biodeterior Biodegradation 18: 110–118. doi: 10.1016/j.ibiod.2017.01.029

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Env Microbiol 72(7):5069–72. doi: 10.1128/AEM.03006-05 [PubMed: 16820507]

Desai JD & Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol R 61: 47–64. doi: 0146-0749/97/$04.0010

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19): 2460–2461. doi: 10.1093/bioinformatics/btq461. [PubMed: 20709691]

Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Department of Genome Sciences, University of Washington, Seattle., Distributed by the author.

Franklin R, Garland J, Bolster C, Mills A. (2001) Impact of dilution on microbial community structure and functional potential: comparison of numerical simulation and batch culture experiments. Appl Environ Microbiol. 67: 702–712. doi: 10.1128/AEM.67.2.702-712.2001 [PubMed: 11157234]

Grund A, Shapiro J, Fennewald M, Bacha P, Leahy J, Markbreiter K, Nieder M & Toepfer M (1975) Regulation of alkane oxidation in Pseudomonas putida. J Bacteriol 123: 546–56. [PubMed: 1150626]

Guibert L, Loviso C, Marcos M, Commendatore M, Dionisi H & Lozada M (2012) Alkane biodegradation genes from chronically polluted subantarctic coastal sediments and their shifts in response to oil exposure. Microb Ecol 64: 605–16. doi: 10.1007/s00248-012-0051-9 [PubMed: 22580956]

Graham EB, Knelman JE, Schindlbacher A & Siciliano S et al. (2016) Microbes as engines of ecosystem function: When does community structure enhance predictions of ecosystem processes? Front Microbiol 7: Article 214. doi: 10.3389/fmicb.2016.00214

Hassanshahian M, Emtiazi G, Kermanshahi RK, et al., (2010) Comparison of oil degrading microbial communities in sediments from Persian Gulf and Caspian Sea. Soil Sediment Contam 19: 277–291. doi: 10.1080/15320381003695215

Hewson I, Vargo GA & Fuhrman JA (2003). Bacterial diversity in shallow oligotrophic marine benthos and overlying waters: effects of virus infection, containment, and nutrient enrichment. Microb Ecol 46: 322–336. doi: 10.1007/s00248-002-1067-3 [PubMed: 14502411]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Huber JA, Welch DBM, Morrison HG, Huse SM, Neal PR, Butterfield DA & Sogin ML (2007) Microbial population structures in the deep marine biosphere. Science 318: 97–100. doi: 10.1126/science.1146689 [PubMed: 17916733]

Ivshina IB, Kuyukina MS, Philp JC & Christofi N (1998) Oil desorption from mineral and organic materials using biosurfactant complexes produced by Rhodococcus species. World J Microb Biot 14: 711–7. doi: 10.1023/A:1008885309221

Joye SB, Kleindienst S, Gilbert JA, Handley KM, Weisenhorn P, Overholt WA & Kostka JE (2016). Responses of microbial communities to hydrocarbon exposures. Oceanography 29(3): 136–149. doi:10.5670/oceanog.2016.78

Kasai Y, Kishira H, Syutsubo K & Harayama S (2001) Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident. Environ Microbiol 3:246–255. doi: 10.1046/j.1462-2920.2001.00185.x [PubMed: 11359510]

Kimes NE, Callaghan AV, Suflita JM & Morris PJ (2014) Microbial transformation of the Deepwater Horizon oil spill – past, present, and future perspectives. Front Microbiol 18(5): Article 603. doi: 10.3389/fmicb.2014.00603. eCollection 2014.

Kloos K, Munch JC & Schloter M (2006) A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. J Microbiol Meth 66: 486–496. doi: 10.1016/j.mimet.2006.01.014

Kok M, Oldenhuis R, van der Linden MP, Raatjes P, Kingma J, van Lelyveld PH & Witholt B (1989) The Pseudomonas oleovorans alkane hydroxylase gene. Sequence and expression. J Biol Chem 264: 5435–41. [PubMed: 2647718]

Koo H; Mojib N; Thacker RW; Bej AK (2014) Comparative analysis of bacterial community metagenomics in coastal Gulf of Mexico sediment microcosms following exposure to Macondo oil (MC252). Antonie Van Leeuwenhoek 106(5): 993–1009. doi: 10.1007/s10482-014-0268-3 [PubMed: 25205599]

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M (2011) Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Appl Environ Microbiol 77: 7962–7974. doi: 10.1128/AEM.05402-11 [PubMed: 21948834]

Kubota M, Nodate M, Yasumoto-Hirose M, Uchiyama T, Kagami O, Shizuri Y & Misawa N (2005) Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments. Biosci Biotechnol Biochem 69: 2421–30. doi: 10.1271/bbb.69.2421 [PubMed: 16377903]

Lamendella R, Strutt S, Borglin S, Chakraborty R, Tas N, Mason OU, Hultman J, Prestat E, Hazen TC, Jansson JK (2014) Assessment of the Deepwater Horizon oil spill impact on gulf coast microbial communities. Front Microbiol 5: Article 130. doi: 10.3389/fmicb.2014.00130

Larkin MJ, Kulakov LA & Allen CCR (2005) Biodegradation and Rhodococcus – masters of catabolic versatility. Curr Opin Biotech 16: 282–90. doi: 10.1016/j.copbio.2005.04.007 [PubMed: 15961029]

Launen LA, Dutta J, Turpeinen R, Eastep ME, Dorn R, Buggs VH, Leonard JW & Häggblom MM (2008) Characterization of the indigenous PAH-degrading bacteria of Spartina dominated salt marshes in the New York/New Jersey Harbor. Biodegradation 19: 347–63. doi: 10.1007/s10532-007-9141-7 [PubMed: 17636392]

Lennon JT (2011) Replication, lies and lesser-known truths regarding experimental design in environmental microbiology. Environ Microbiol 13(6): 1383–1386. doi:10.1111/j.14622920.2011.02445.x [PubMed: 21392200]

Liu Z, & Liu J (2013) Evaluating bacterial community structures in oil collected from the sea surface and sediment in the northern Gulf of Mexico after the Deepwater Horizon oil spill. MicrobiologyOpen 2: 492–504. doi: 10.1002/mbo3.89 [PubMed: 23568850]

Liu C, Wang W, Wu Y, Zhou Z, Lai Q & Shao Z (2011) Multiple alkane hydroxylase systems in a marine alkane degrader, Alcanivorax dieselolei B-5. Environ Microbiol 13: 1168–78. doi: 10.1111/j.1462-2920.2010.02416.x [PubMed: 21261799]

Looper JK, Cotto A, Kim B-Y, Lee M-K, Liles MR, Ní Chadhain SM & Son A (2013) Microbial community analysis of Deepwater Horizon oil-spill impacted sites along the Gulf coast using

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

functional and phylogenetic markers. Env Sci Process Impact 15: 2068–79. doi: 10.1039/c3em00200d

Lozupone C & Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71: 8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005 [PubMed: 16332807]

Liu Q, Tang J, Bai Z, Hecker M & Giesy J (2015) Distribution of petroleum degrading genes and factor analysis of petroleum contaminated soil from the Dagang Oilfield, China. Sci Rep 5: Article 11068. doi: 10.1038/srep11068.

Lu Z, Deng Y, Van Nostrand JD, He Z, Voordeckers J, Zhou A, et al. (2012). Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. ISME J. 6: 451–460. doi: 10.1038/ismej.2011.91 [PubMed: 21814288]

Magnusson M C Colgan & Gittell R (2012). The economic impact of the Piscataqua River and the Ports of Portsmouth and Newington. State of New Hampshire Division of Ports and Harbors. http://www.portofnh.org/documents/port_study_mm_6_7_12FINAL.pdf

Mahmoudi N; Porter TM; Zimmerman AR; Fulthorpe RR; Kasozi GN; Silliman BR; Slater GF (2013) Rapid degradation of Deepwater Horizon spilled oil by indigenous microbial communities in Louisiana saltmarsh sediments. Environ. Sci. Technol 47 (23): 13303–13312. doi: 10.1021/es4036072 [PubMed: 24219093]

Maier T, Forster HH, Asperger O & Hahn U (2001) Molecular characterization of the 56-kDa CYP153 from Acinetobacter sp. EB104. Biochem Biophys Res Commun 286: 652–8. doi: 10.1006/bbrc.2001.5449 [PubMed: 11511110]

Makepeace DK, Smith DW & Stanley SJ (1995) Urban stormwater quality: Summary of contaminant data. Crit Rev Env Sci Tech 25: 93–139. doi: 10.1080/10643389509388476

Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, Han J, Holman HY, Hultman J, Lamendella R, Mackelprang R, Malfatti S, Tom LM, Tringe SG, Woyke T, Zhou J, Rubin EM, Jansson JK (2012) Metagenome, metatranscriptome and singlecell sequencing reveal microbial response to Deepwater Horizon oil spill. ISME J 6:1715–27. doi: 10.1038/ismej.2012.59. [PubMed: 22717885]

McGenity TJ (2014). Hydrocarbon biodegradation in intertidal wetland sediments. Curr. Opin. Biotechnol 27: 46–54. 10.1016/j.copbio.2013.10.010 [PubMed: 24863896]

Morgan Pamela A.; Burdick David M.; and Short Frederick T., The Functions And Values Of Fringing Salt Marshes In Northern New England, USA (2009). Estuaries and Coasts 32(3): 483–495. doi: 10.1007/s12237-009-9145-0

Natter M, Keevan J, Wang Y, Keimowitz AR, Okeke BC, Son A, Lee MK. (2012) Level and degradation of Deepwater Horizon spilled oil in coastal marsh sediments and pore-water. Environ. Sci. Technol 46 (11): 5744–5755. doi: 10.1021/es300058w [PubMed: 22571231]

National Research Council, 2007 The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet. Washington, DC: The National Academies Press https://doi.org/10.17226/11902

NHDES, 2004 Environmental Fact Sheet WMB-CP-08. Threats to the Salt Marsh Environment. Available at: https://www.des.nh.gov/organization/commissioner/pip/factsheets/cp/documents/cp-08.pdf

NHDES. 2015 State of New Hampshire DRAFT 2014 Section 303(d) Surface Water Quality List. NHDES-R-WD-15–11. October 14, 2015. New Hampshire Department of Environmental Services, Concord, NH.

Nie Y, Chi C-Q, Fang H, Liang J-L, Lu S-L, Lai G-L, Tang Y-Q & Wu XL. (2014) Diverse alkane hydroxylase genes in microorganisms and environments. Sci Rep 4: Article 4968 doi: 10.1038/srep04968.Sa

Paisse S, Goni-Urriza M, Coulon F & Duran R (2010) How a bacterial community originating from a contaminated coastal sediment responds to an oil input. Microb Ecol 60: 394–405. doi: 10.1007/s00248-010-9721-7 [PubMed: 20652237]

Pérez-de-Mora A, Engel M & Schloter M (2011) Abundance and diversity of n-alkane-degrading bacteria in a forest soil co-contaminated with hydrocarbons and metals: a molecular study on alkB homologous genes. Microbial Ecol 62: 959–972. doi: 10.1007/s00248-011-9858-z

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Pi Y, Chen B, Bao M, Fan F, Cai Q, Ze L & Zhang B (2017) Microbial degradation of four crude oil by biosurfactant producing strain Rhodococcus sp. Bioresour Technol 232: 263–269. doi: 10.1016/j.biortech.2017.02.007. [PubMed: 28236759]

Pielou EC (1977) Mathematical ecology. New York: Wiley.

Pornsunthorntawee O, Wongpanit P, Chavadej S, Abe M & Rujiravanit R (2008) Structural and physicochemical characterization of crude biosurfactant produced by Pseudomonas aeruginosa SP4 isolated from petroleum-contaminated soil. Bioresource Technol 99: 1589–95. doi: 10.1016/j.biortech.2007.04.020

Piscataqua River Estuaries Partnership (PREP) (2013) State of Our Estuaries. University of New Hampshire, Dover, NH pp. 48

Price MN, Dehal PS, Arkin AP. 2010 FastTree 2-Approximately Maximum-Likelihood Trees for large alignments. Plos One 5(3). doi: 10.1371/journal.pone.0009490

Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-González MI, Rojas A, Teran W & Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56: 743–68. doi: 10.1146/annurev.micro.56.012302.161038 [PubMed: 12142492]

Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 11: 2477–90. doi: 10.1111/j.14622920.2009.01948.x [PubMed: 19807712]

Rosenberg M & Rosenberg E (1981) Role of adherence in growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. J Bacteriol 148: 51–7 [PubMed: 7287631]

Sabirova JS, Ferrer M, Regenhardt T, Timmis KN & Golyshin PM (2006) Proteomic insights into metabolic adaptations in Alcanivorax borkumensis induced by alkane utilization. J Bacteriol 188(11): 3763–3773. doi: 10.1128/JB.00072-06 [PubMed: 16707669]

Saul DJ, Aislabie JM, Brown CE, Harris L & Foght JM (2005) Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. FEMS Microbiol Ecol 53: 141–55. doi: 10.1016/j.femsec.2004.11.007: [PubMed: 16329936]

Schafer H, Servais P, and Muyzer G (2000). Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. Arch. Microbiol. 173: 138–145. doi: 10.1007/s002039900121 [PubMed: 10795685]

Schloss P & Handelsman J (2009) Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–41. doi: 10.1128/AEM.01541-09 [PubMed: 19801464]

Schneiker S, dos Santos VAPM, Bartels D et al., (2006) Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium Alcanivorax borkumensis. Nat Biotech 24: 997–1004. doi: 10.1038/nbt1232

Sekine M, Tanikawa S, Omata S et al., (2006) Sequence analysis of three plasmids harboured in Rhodococcus erythropolis strain PR4. Environ Microbiol 8: 334–46. doi: 10.1111/j.14622920.2005.00899.x [PubMed: 16423019]

Smits THM, Balada SB, Witholt B & van Beilen JB (2002) Functional analysis of alkane hydroxylases from Gram-negative and Gram-positive bacteria. J Bacteriol 184: 1733–42. doi: 10.1128/JB.184.6.1733-1742.2002 [PubMed: 11872725]

Speight JG (1998) The Chemistry and Technology of Petroleum: 3 Edition. New York, NY, USA: Marcel Dekker Inc.

Stralberg D, Brennan M, Callaway JC, Wood JK, Schile LM, et al., (2011) Evaluating tidal marsh sustainability in the face of sea–level rise: A hybrid modeling approach applied to San Francisco Bay. PLoS One 6 (11): e27388 doi:10.1371/journal.pone.0027388 [PubMed: 22110638]

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 10: 2731–9. doi: 10.1093/molbev/msr121

Teeling H & Glöckner FO (2012) Current opportunities and challenges in microbial metagenome analysis: a bioinformatic perspective. Brief Bioinform 13(6): 728–742. doi:10.1093/bib/bbs039 [PubMed: 22966151]

van Beilen JB & Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Applied Microbiol Biotechnol 74: 13–21. doi: 10.1007/s00253-006-0748-0

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

van Beilen JB, Funhoff EG, van Loon A et al., (2006) Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Appl Environ Microbiol 72: 59–65. doi: 10.1128/AEM.72.1.59-65.2006 [PubMed: 16391025]

van Beilen JB, Kingma J & Witholt B (1994) Substrate specificity of the alkane hydroxylase system of Pseudomonas oleovorans GPo1. Enzyme Microb Tech 16: 904–11. doi: 10.1016/0141-0229(94)90066-3

van Beilen JB, Smits THM, Roos FF, Brunner T, Balada SB, Röthlisberger M & Witholt B (2005) Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. J Bacteriol 187: 85–91. doi: 10.1128/JB.187.1.85-91.2005 [PubMed: 15601691]

van Hamme JD & Ward OP (2001) Physical and metabolic interactions of Pseudomonas sp. strain JA5B45 and Rhodococcus sp. strain F9-D79 during growth on crude oil and effect of a chemical surfactant on them. Appl Environ Microbiol 67: 4874–9. doi: 10.1128/AEM. 67.10.4874-4879.2001 [PubMed: 11571196]

Vazquez-Baeza Y, Pirrung M, González A & Knight R (2013) EMPeror: a tool for visualizing highthroughput microbial community data. GigaScience 2: 16. doi: 10.1186/2047-217X-2-16 [PubMed: 24280061]

Vega FA, Covelo EF, Reigosa MJ, Andrade ML (2009) Degradation of fuel oil in saltmarsh soils affected by the Prestige oil spill. J Hazard Mater 166:1020–9 doi: 10.1016/j.jhazmat.2008.11.113. [PubMed: 19157704]

Vermuë M, Sikkema J, Verheul A, Bakker R & Tramper J (1993) Toxicity of homologous series of organic solvents for the gram-positive bacteria Arthrobacter and Nocardia sp. and the gramnegative bacteria Acinetobacter and Pseudomonas sp. Biotechnol Bioeng 42: 747–58. doi: 10.1002/bit.260420610 [PubMed: 18613108]

Vieites DR, Nieto-Roman S, Palanca A, Ferrer X, Vences M (2004) European Atlantic: the hottest oil spill hotspot worldwide. Naturwissenschaften 91: 535–538. doi: 10.1007/s00114-004-0572-2 [PubMed: 15490095]

Vieira-Silva S, Rocha EPC (2010) The systemic imprint of growth and its uses in ecological (meta)genomics. PLoS Genet 6(1): e1000808. doi: 10.1371/journal.pgen.1000808 [PubMed: 20090831]

Walker JD & Colwell RR (1975) Some effects of petroleum on estuarine and marine microorganisms. Can J Microbiol 21: 305–313. [PubMed: 1116043]

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb 73(16): 5261–5267. doi: 10.1128/AEM.00062-07

Wang W & Shao Z (2013) Enzymes and genes involved in aerobic alkane degradation. Front Microbiol 4: 116. doi: 10.3389/fmicb.2013.00116 [PubMed: 23755043]

Wang L, Wang W, Lai Q & Shao Z (2010a) Gene diversity of CYP153A and alkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean. Environ Microbiol 12: 1230–42. doi: 10.1111/j.1462-2920.2010.02165.x. [PubMed: 20148932]

Wang W, Wang L & Shao Z (2010b) Diversity and abundance of oil-degrading bacteria and alkane hydroxylase (alkB) genes in the subtropical seawater of Xiamen Island. Microbial Ecol 60: 429–39. doi: 10.1007/s00248-010-9724-4

Watts AW, Ballestero TP & Gardner KH (2006) Uptake of polycyclic aromatic hydrocarbons (PAHs) in salt marsh plants Spartina alterniflora grown in contaminated sediments. Chemosphere 62: 1253–60. doi: 10.1016/j.chemosphere.2005.07.006 [PubMed: 16213549]

Wentzel A, Ellingsen T, Kotlar H-K, Zotchev S & Throne-Holst M (2007) Bacterial metabolism of longchain n-alkanes. Appl Microbiol Biotech 76: 1209–21. doi: 10.1007/s00253-007-1119-1

Whyte LG, Hawari J, Zhou E, Bourbonnière L, Inniss WE & Greer CW (1998) Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic Rhodococcus sp. Appl Environ Microbiol 64: 2578–84. [PubMed: 9647833]

Whyte LG, Smits THM, Labbé D, Witholt B, Greer CW & van Beilen JB (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in Rhodococcus strains Q15 and NRRL

B-16531. Appl Environ Microbiol 68: 5933–42. doi: 10.1128/AEM.68.12.5933-5942.2002 [PubMed: 12450813]

Wigand C, McKinney RA, Chintala MM, Charpentier MA & Groffman PM (2004) Denitrification Enzyme Activity of Fringe Salt Marshes in New England (USA). J of Envt Qual 33(3): 11441151. doi: 10.2134/jeq2004.1144

Xu JL, He J, Wang ZC, Wang K, Li WJ, Tang SK & Li SP (2007) Rhodococcus qingshengii sp. nov., a carbendazim-degrading bacterium. Int J Syst Evol Microbiol 57: 2754–2757. doi: 10.1099/ijs.0.65095-0 [PubMed: 18048720]

Yan Y, Kuramae EE, Klinkhamer PGL, van Veen JA. (2015). Revisiting the dilution procedure used to manipulate microbial biodiversity in terrestrial systems. Appl Environ Microbiol 81: 4246–4252. doi: 10.1128/AEM.00958-15 [PubMed: 25888175]

Zhu X, Venosa AD, Suidan MT & Lee K (2004) Guidelines for the Bioremediation of Oil-Contaminated Salt Marshes. Cincinnati, OH, US Environmental Protection Agency National Risk Management Research Laboratory Office of Research and Development pp. 61

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Highlights

- A fringing tidal marsh microbial community is dominated by Beta- and Gammaproteobacteria.
- Pseudomonad and Rhizobiales type *alkB* and *CYP 153A1* alkane hydroxylase genes were detected.
- Pseudomonads dominated communities enriched with shorter chain n-alkanes.
- Rhodococci dominated a community enriched with dodecane.
- *AlkB* alkane hydroxylases were more abundant than *CYP 153A1* hydroxylases in marsh sediments.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

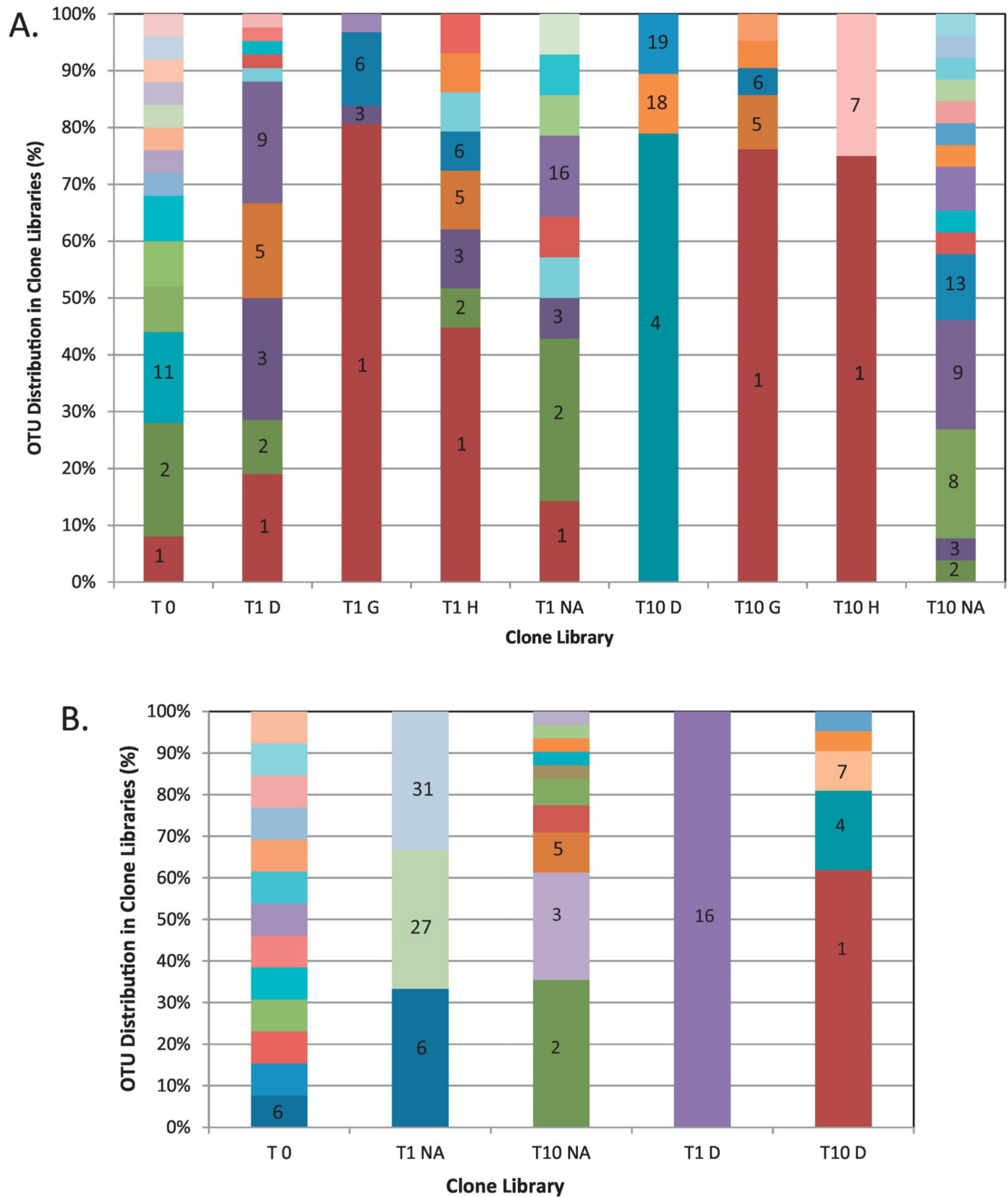

Fig. 1.

The distribution of *alkB* (A) and *P450* (B) OTUs in clone libraries derived from initial (T0), 1 day (T1) and 10 day (T10) dilution culture samples exposed to dodecane (D), gasoline (G), *n*-hexane (H) or no alkanes (NA – control). Alignments, generation of distance matrices and clustering into OTUs at a similarity level of 95% was performed as described in the Methods. OTU numbering is included for OTUs that occurred in >10% of the total recovered for that library. See Table 2 for further information on OTU identity and Table 1 for the number of clone sequences in total for each library shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

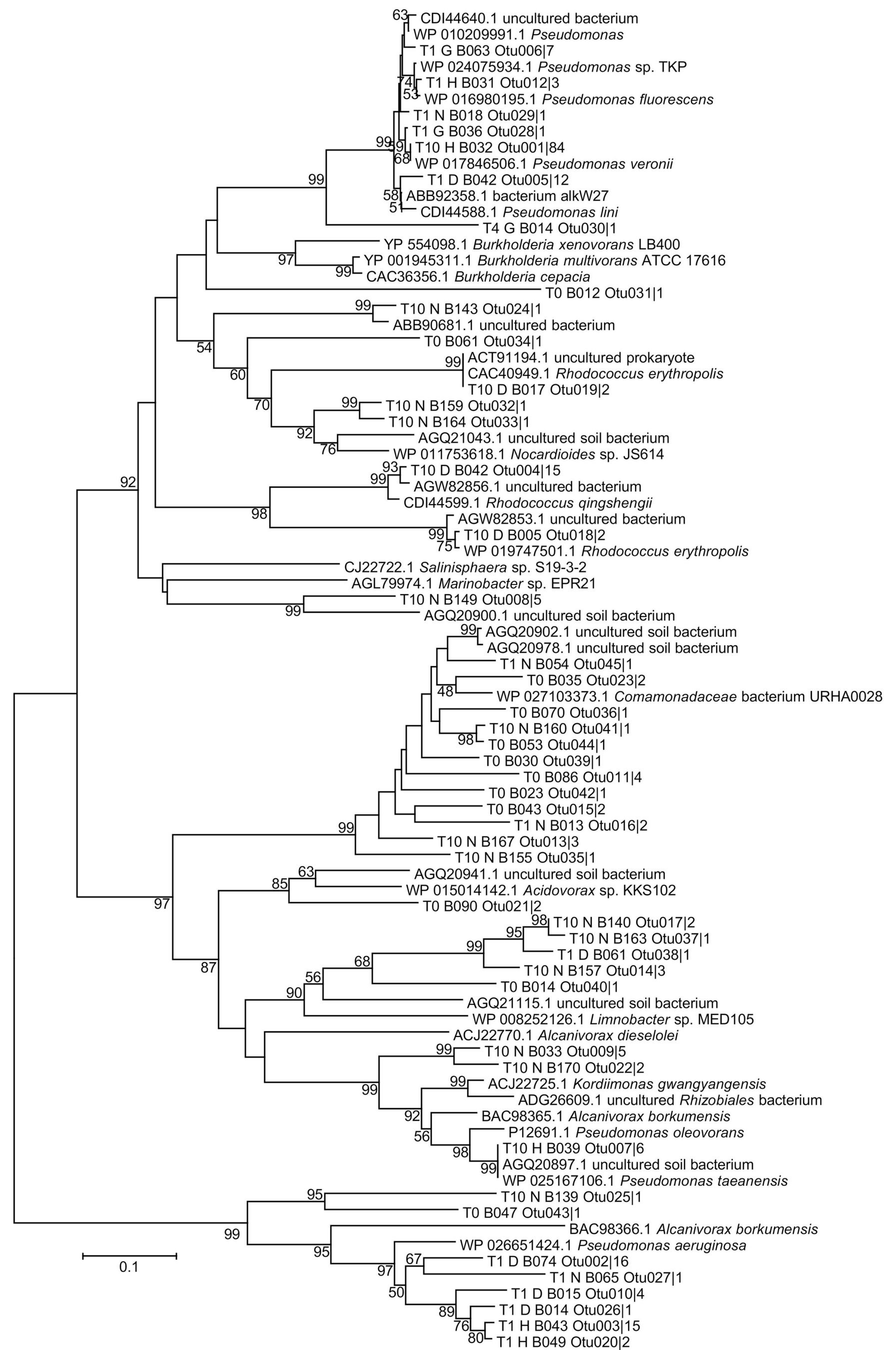

Fig. 2.

Phylogenetic distribution of *alkB* sequences. The phylogenetic tree was constructed from a ClustalW alignment of the derived amino acid sequences by neighbor-joining analysis with 1000 bootstrap replicates using Mega5.0 (Tamura et al. 2011). Bootstrap values greater than 50 are indicated. T0, T1 and T10 indicate zero (initial), 1 day and 10 day dilution culture samples. Alkanes are indicated as D (dodecane), H (hexane), G (gasoline) or NA (no alkane control).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

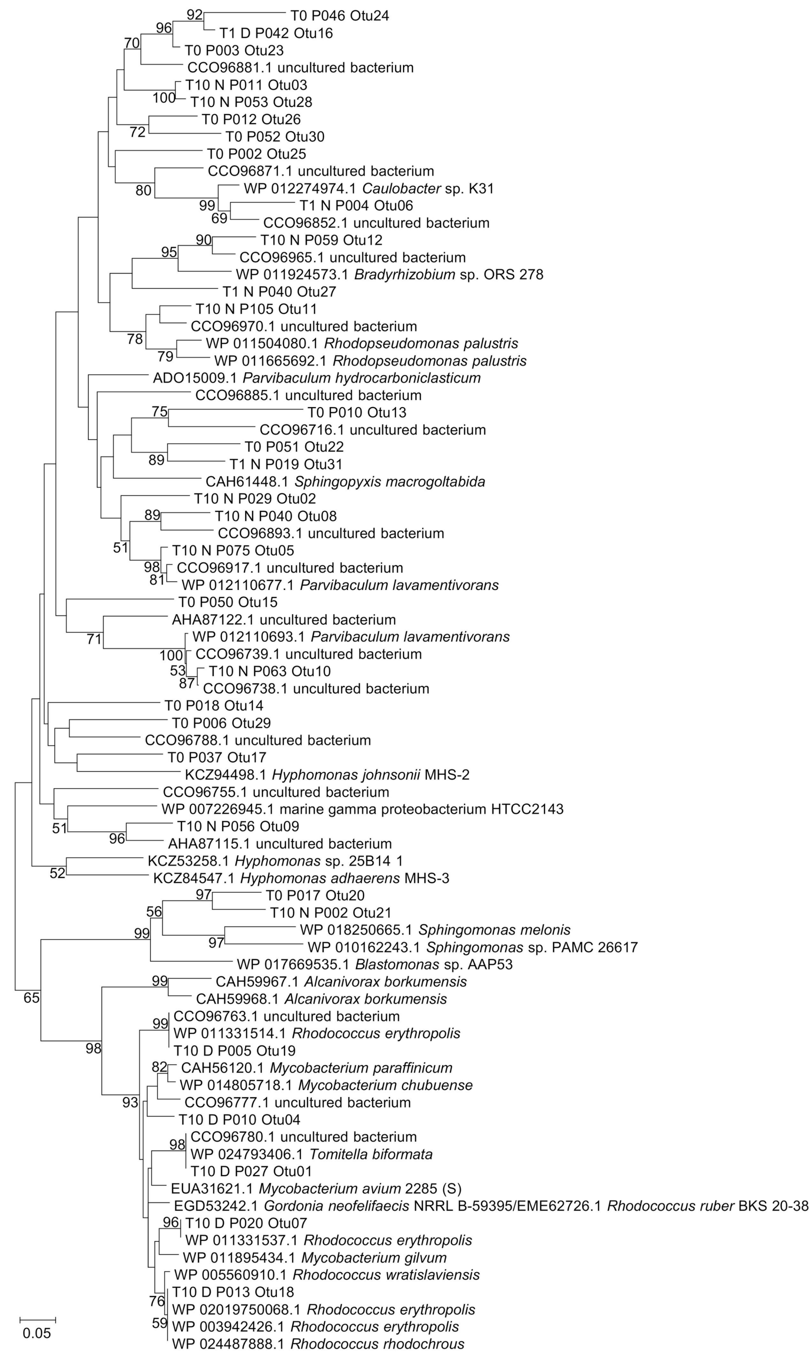

Fig. 3.

Phylogenetic distribution of *P450 CYP153A1* sequences. The phylogenetic tree was constructed from a ClustalW alignment of the derived amino acid sequences by neighbor-joining analysis with 1000 bootstrap replicates using Mega5.0 (Tamura et al. 2011). Bootstrap values greater than 50 are indicated. T0, T1 and T10 indicate zero (initial), 1 day and 10 day dilution culture samples. Alkanes are indicated as D (dodecane), H (hexane), G (gasoline) or NA (no alkane control).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

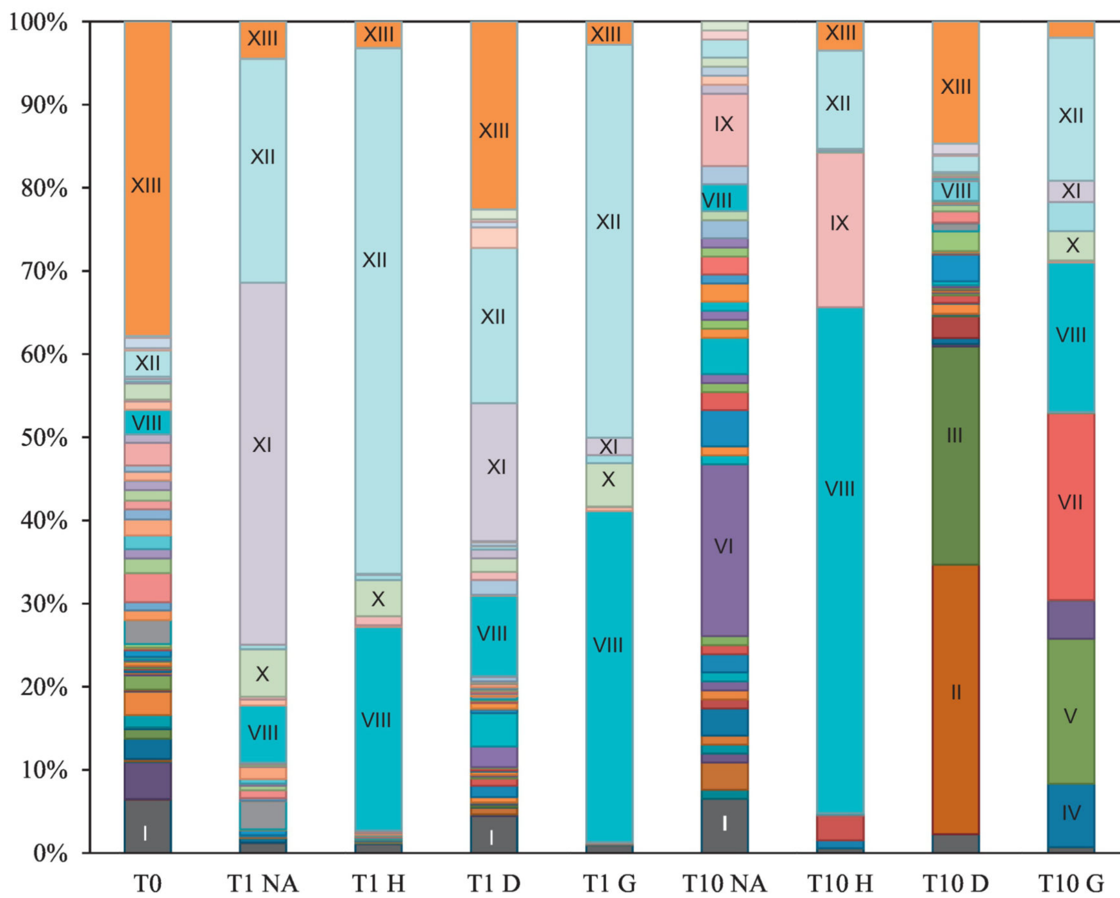

Fig. 4.

Bar graph showing taxonomic distribution of tags into OTUs at the genus, family and class level. OTUs accounting for greater than 5% of the sequences within a sample are labeled as follows: I, Other; II, Genus *Mycobacterium*; III, Genus *Rhodococcus*; IV, Genus *Bacillus*; V, Genus *Lysinibacillus*; VI, Family Aurantimonadaceae; VII, Family Acetobacteraceae; VIII, Class Gammaproteobacteria; IX, Genus *Marinobacter*; X, Genus *Shewanella*; XI, Genus *Acinetobacter*; XII, Genus *Pseudomonas*; XIII, groups that account for <1% of any dilution culture sample. T0, T1 and T10 indicate zero (initial), 1 day and 10 day dilution culture samples. Alkanes are indicated as D (dodecane), H (hexane), G (gasoline) or NA (no alkane control).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

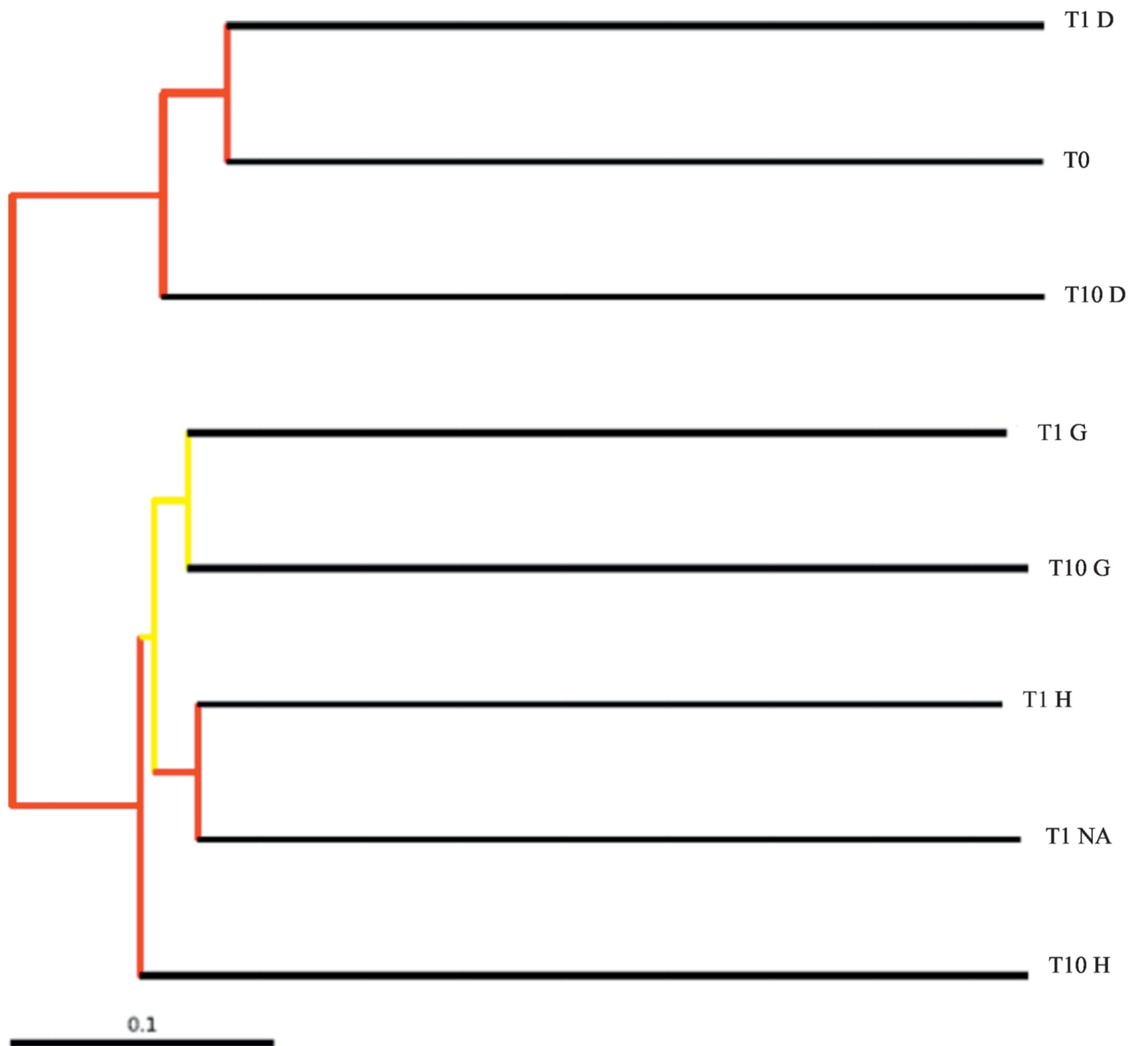

Fig. 5.

Dendrogram of hierarchical clustering of community similarities from all libraries (excluding the T10 NA due to low number of sequences recovered) based on weighted UniFrac Jackknife Cluster Analysis with 100 permutations (5000 sequence sub-sampling). Each node is colored by the fraction of times it was recovered in the jackknife replicates. Nodes recovered >99.9% of the time are red, 90–99.9% are yellow. T0, T1 and T10 indicate zero (initial), 1 day and 10 day dilution culture samples. Alkanes are indicated as D (dodecane), H (hexane), G (gasoline) or NA (no alkane control).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

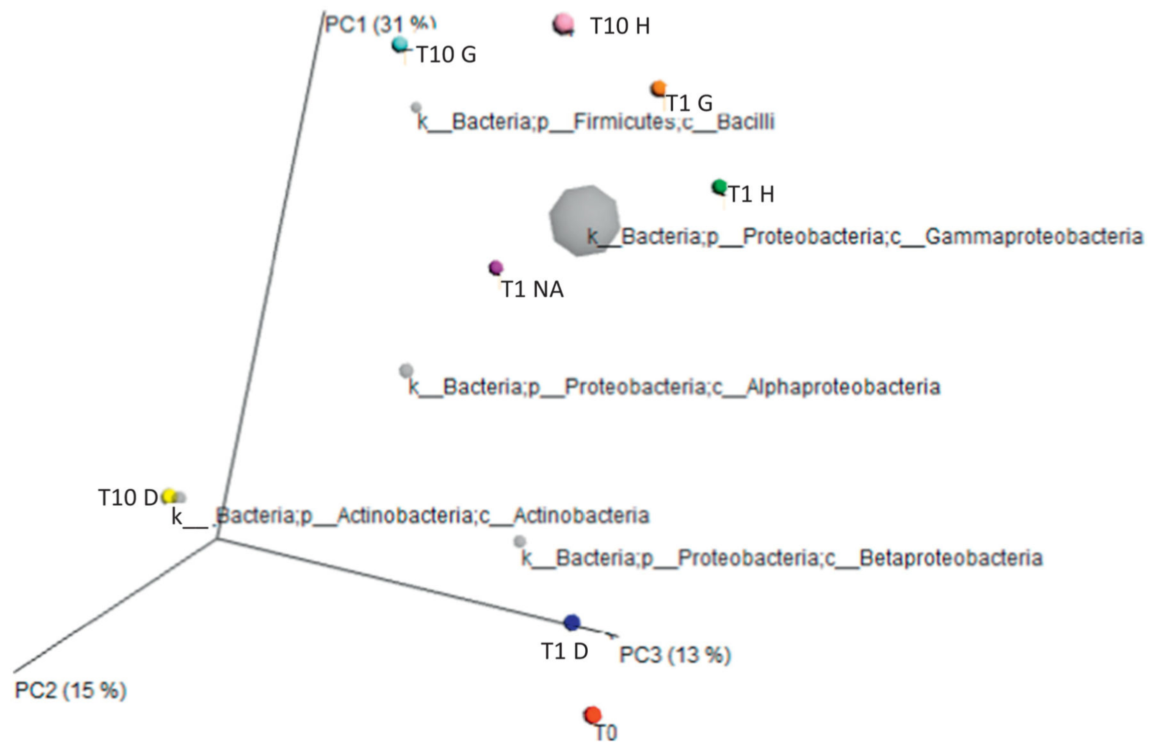

Fig. 6.

Principal coordinate analysis (PCoA) ordination based on an unweighted UniFrac distance matrix of sediment bacterial 16S rDNA gene profiles sampled from the initial sediment (T0), and after 1 and 10 days of exposure to dodecane (D), hexane (H) and gasoline (G) and no alkane control (NA). The T10 day no alkane control sample was not included due to the low number of sequences recovered. K ordination value labels indicate p = phylum and c = class.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Table 1.

Diversity analysis of functional gene clone libraries. OTUs were clustered at the 95% identity level using the furthest neighbor in Mothur version 1.33.3

Library	N	N	H’	Chao1
alkB				
TO	25	14	2.44	23
T1 NA	14	9	2.04	14
T1 H	29	8	1.75	8
T1 D	33	9	1.81	19
T1 G	31	4	0.65	5
T10 NA	26	15	2.46	42.5
T10 H	24	2	0.56	2
T10 D	19	3	0.66	3
T10 G	21	5	0.87	6.5
CYP 153A1				
TO	13	13	2.56	91
T1 NA	3	3	1.1	1
T1 D	21	1	0	6
T10 NA	31	10	1.85	13.3
T10 D	1	5	1.13	5.5

[a]*N*, number of clones sequenced from each library; *n*, number of OTUs observed; H′, Shannon-Weaver diversity index, Chao1 nonparametric richness estimate. Numbers in parentheses represent 95% confidence intervals; ND, not determined. Libraries are labelled as: initial sediment (T0), 1 day (T1) and 10 day (T10) dilution culture samples, with alkane exposure either dodecane (D), gasoline (G), *n*-hexane (H) or no alkanes (NA – control).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2.

Best BlastX matches (by identity) in the GenBank database for the dominant alkane hydroxylase clone OTUs. Where best match has no taxonomic information the first match with taxonomic information is also included.

OTU # | # of sequences in OTU[1]	Gene ID	Name	Identity (%)	e-value
alkB				
OTU1|84	WP_017846506.1	*Pseudomonas veronii*	100	3E-114
OTU2|16	WP_026651424.1	*Pseudomonas aeruginosa*	81	3E-75
OTU3|15	WP_026651424.1	*Pseudomonas aeruginosa*	84	3E-87
OTU4|15	ABB90689.1	uncultured bacterium	99	8E-116
	AID55567.1	*Rhodococcus* sp. RP-11	99	3E-115
OTU5|12	CDI44588.1	*Pseudomonas lini*	96	6E-113
OTU6|7	WP_038843879.1	*Pseudomonas fluorescens*	99	2E-113
OTU7|6	ACT91140.1	uncultured bacterium	100	1E-119
	ADG26619.1	uncultured Rhizobiales bacterium	100	2E-119
OTU8|5	AGQ20900.1	uncultured bacterium	80	9E-91
	ACJ22711.1	*Alcanivorax* sp. S15–9	67	3E –80
OTU9|5	ACJ22725.1	*Kordiimonas gwangyangensis*	81	5E-85
OTU11|4	AGQ20978.1	uncultured bacterium	83	3E-95
	WP_027103373.1	*Comamonadaceae bacterium URHA0028*	84	7E-94
OTU13|3	AGQ20902.1	uncultured bacterium	82	3E –90
	WP_027103373.1	*Comamonadaceae* bacterium URHA0028	81	7E –89
OTU16|2	ABB96070.1	uncultured bacterium	86	1E –96
	WP_027103373.1	*Comamonadaceae* bacterium URHA0028	85	1E-93
OTU18|2	WP_030535917.1	*Rhodococcus erythropolis*	100	4E-112
OTU19|2	BAG06232.1	*Rhodococcus erythropolis*	99	7E-117
CYP 153A1				
OTU1|13	CCO96780.1	uncultured bacterium	100	4E-64
	WP_032375608.1	*Rhodococcus fascians*	100	3E-63
OTU2|11	ADF27209.1	uncultured Rhizobiales bacterium	93	2E-60
OTU3|8	AKG58848.1	uncultured bacterium	88	2E-56
	ADO15009.1	*Parvibaculum hydrocarboniclasticum*	80	7E-51
OTU4|4	ADF27251.1	uncultured Rhizobiales bacterium	93	2E-61
OTU5|3	CCO96917.1	uncultured bacterium	98	4E-63
	ACP39704.1	*Parvibaculum* sp. S18–4	97	7E-62
OTU6|2	ADF27232.1	uncultured Rhizobiales bacterium	94	3E-48
OTU7|2	CAH56117.1	*Rhodococcus erythropolis*	99	2E-64
OTU16|1	CCO96881.1	uncultured bacterium	86	5E-55
	ADF27267.1	uncultured Rhizobiales bacterium	99	2E-53
OTU27|1	ABI14020.1	*Rhodococcus* sp. MOP100	87	1E-54
OTU31|1	AKG58846.1	uncultured bacterium	78	1E-49

OTU # | # of sequences in OTU[1]	Gene ID	Name	Identity (%)	e-value
	ADO15009.1	*Parvibaculum hydrocarboniclasticum*	77	5E-49

[1]The number of matches to this OTU relative to all of the clone sequences from all libraries is shown. For example, there were 84 clone library sequences that comprised OTU1 when all libraries were considered.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 3.

QPCR analysis of selected alkane hydroxylase OTU DNA. Standard deviations are included in parentheses.

OTU	Abundance of OTU as: Copy # of OTU DNA/ng gDNA / Copy # 16S rDNA/ng gDNA								
	Sample[a]								
AlkB									
	T0	**T1 NA**	**T10 NA**	**T1 D**	**T10 D**	**T1 H**	**T10 H**	**T1 G**	**T10 G**
1	ND	ND	ND	0.05 (±0.08)	0.02 (±0.02)	0.40 (±0.33)	3.00 (±2.01)	0.33 (±0.27)	0.48 (±0.55)
2	ND	1.30 (±0.83)	ND	0.21 (±0.29)	ND	1009 (±873.83)	0.02 (±0.02)	ND	71 (±113.85)
3	ND	0.74 (±0.42)	ND	2.36 (±1.18)	0.64 (±0.14)	2.88 (±1.49)	0.59 (±0.18)	0.04 (±0.01)	0.08 (±0.00)
4	ND	ND	ND	ND	0.57 (±1.79)	ND	ND	5.95 (±14.61)	ND
5	0.05 (±0.01)	0.03 (±0.02)	ND	0.02 (±0.02)	0.03 (±0.01)	0.06 (±0.04)	0.01 (±0.01)	0.03 (±0.01)	0.02 (±0.03)
CYP 153A1									
1	ND	ND	ND	ND	0.47 (±0.68)	ND	ND	ND	ND
4	ND	ND	ND	ND	0.10 (±0.23)	ND	ND	ND	ND
7	ND	ND	ND	ND	0.31 (±0.81)	ND	ND	ND	ND
16	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]Sample names are labelled as: initial sediment (T0), 1 day (T1) and 10 day (T10) dilution culture samples, with alkane exposure either dodecane (D), gasoline (G), *n*-hexane (H) or no alkanes (NA – control).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 4.

Summary of IonTorrent PGM 16S rDNA V6 tag sequencing efforts and alpha diversity metrics. Standard deviations are included in parentheses.

Library	Barcode	Processed sequences	OTUs observed	Chao1	Shannon Index	Phylogenetic Distance
T0	ATCAG	68,610	2132	4942 (216)	10.0 (0.01)	75
T1 NA	GTGAG	23,508	528	1551 (201)	5.0 (0.1)	34
T1 H	GATCT	45,242	426	1274 (145)	4.4 (0.0)	28
T1 D	CGTCT	13,730	1736	5274 (344)	8.4 (0.0)	61
T1 G	CGACG	62,669	346	921 (70)	3.9 (0.0)	23
T10 NA	AGCAT	92	nd	nd	nd	nd
T10 H	TGTCA	9,794	407	407 (38)	2.5 (0.0)	16
T10 D	GCGAT	32,439	782	2075 (246)	5.5 (0.0)	42
T10 G	TCTGT	25,689	872	872 (106)	4.5 (0.0)	22

Libraries are labelled as initial sediment (T0), 1 day (T1) and 10 day (T10) dilution culture samples, with alkane exposure either dodecane (D), gasoline (G), *n*-hexane (H) or no alkanes (NA – control).