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

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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 h 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 gram-positive bacteria. The data demonstrate that small fringing marshes in New England harbor petroleum-degrading bacteria, suggesting that petroleum degradation may be an important fringing marsh ecosystem function.

1. 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 hydrocarbon-degrading 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., 2014; 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 (Acosta-González 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 and Funhoff, 2007; Wang et al., 2010a, 2010b). 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 and 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.,

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2012; van Beilen and 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 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 (NHDES, 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.

2. Materials and methods

2.1. 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 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 × 3 m area containing vegetated and un-vegetated areas. These were composited into a sterile 1 gal 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 h 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.

2.2. 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 1 μM of forward and reverse primers, 2× 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 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final elongation of 72 °C for 5.5 min.

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