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Enhancing the biodegradation of oil in sandy sediments with choline: A naturally methylated nitrogen compound

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ABSTRACT

We investigated how additions of choline, a naturally occurring methylated nitrogen-containing compound, accelerated hydrocarbon degradation in sandy sediments contaminated with moderately weathered crude oil (4000 mg kg^{-1} sediment). Addition of lauroylcholine chloride (LCC) and tricholine citrate (TCC) to oil contaminated sediments resulted in 1.6 times higher hydrocarbon degradation rates compared to treatments without added choline derivatives. However, the degradation rate constant for the oil contaminated sediments amended with LCC was similar to that in contaminated sediments amended with inorganic nitrogen, phosphorus, and glucose. Additions of LLC and TCC to sediments containing extensively weathered oil also resulted in enhanced mineralization rates. Cultivation-free 16S rRNA analysis revealed the presence of an extant microbial community with clones closely related to known hydrocarbon degraders from the *Gammaproteobacteria*, *Alphaproteobacteria*, and *Firmicutes* phyla. The results demonstrate that the addition of minimal amounts of organic compounds to oil contaminated sediments enhances the degradation of hydrocarbons.

1. Introduction

Following an oil spill, physical clean up of coastal sediments is often a first response intended to minimize the environmental impact. This approach, however, can leave residual hydrocarbons in the environment that can persist for decades (Li and Boufadel, 2010). Bioremediation, or the degradation of contaminants by microorganisms, is an alternative to physical cleaning activities used to enhance *in situ* hydrocarbon degradation. Three approaches have been used to enhance the rate of hydrocarbon biodegradation: (1) stimulation of indigenous microorganisms by the introduction of nutrients and/or oxygen (biostimulation) (Seklemova et al., 2001), (2) inoculation of sediments with an enriched microbial consortium (bioaugmentation) (Barathi and Vasudevan, 2001; Richard and Vogel, 1999), and (3) application of dispersants to oil contaminated areas (Kujawinski et al., 2011; Atlas and Hazen, 2011).

Additions of inorganic N and P fertilizers have been effective in enhancing the degradation of oil in beaches (Bragg et al., 1994) and in wetlands (Horel et al., 2012a,b; Jackson and Pardue, 1999, 1997), but the doses that have to be applied to achieve maximum mineralization of the oil appears to be site specific and influenced by the oil type, as well as the degree of oil weathering (Venosa et al., 1996; Jackson and Pardue, 1997; Fernandez-Alvarez et al., 2006). Attempts have also been made to use oleophilic fertilizers that allow the nutrients to remain in the oil phase or slow release fertilizers that can persist in the environment for long periods of time to enhance bioremediation, both of which have resulted in variable degrees of enhanced biodegradation (Fernandez-Alvarez et al., 2006; Jimenez et al., 2006; Choi et al., 2002). As an alternative to addition of inorganic fertilizers, naturally occurring organic matter has been used for enhancing the degradation of oil. Minimal amounts of organic matter, when added to oil contaminated sediments, can significantly increase hydrocarbon degradation rates (Mortazavi et al., 2013; Proctor et al., 2001).

Dispersants have also been used as means of accelerating biodegradation (Atlas and Hazen, 2011). Dispersants consist of surfactants and hydrocarbon-based solvents mixtures. The use decreases the interfacial tension between oil and water and reduces the size of oil droplets (Lee and de Mora, 1999). By inhibiting the formation of large emulsions, dispersants effectively result in droplets with relatively larger surface areas that are more

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accessible to the microbial community. A commercial dispersant, Corexit 9500, was extensively used (Spier et al., 2013) following the explosion of the Deepwater Horizon platform and the ensuing oil spill in the Gulf of Mexico. Application of dispersants at the wellhead resulted in a dispersed oil plume at approximately 1100 m depth that was degraded within several months (Atlas and Hazen, 2011). While the exact composition of Corexit 9500 is not known, sulfosuccinate ester (dioctyl sodium sulfosuccinate, DOSS) and oxyalkylated C_{12}–C_{15} alcohols are key anionic surfactant ingredients in many dispersants (Sterling et al., 2004). Previous laboratory studies have shown that several aquatic species exhibit low to moderate toxicity to Corexit® 9500 and Corexit® 9527 (George-Ares and Clark, 2000) and bacterial isolates from beach sands contaminated with oil from the Deepwater Horizon oil spill demonstrated a reduction in cell viability when exposed to Corexit® 9500 (Hamdan and Fulmer, 2011). The potential toxicity and long-term effects of dispersants on aquatic organisms remain *issues of concern* (Wise and Wise, 2011; Hansen et al., 2012; Rico-Martinez et al., 2013).

We report herein the use of two choline derivatives (tricholine citrate [TCC], and lauroylcholine chloride [LCC] (Fig. 1)) for hydrocarbon bioremediation as alternatives to synthetic surfactants. The potential advantages of choline derivatives for use in remediation of oil spills over commercial surfactants is that choline is (i) a common constituent of eukaryotic cell membranes in the form of phosphatidylcholine (Lehninger, 1975), (ii) present in seawater at nanomolar concentrations (Roulier et al., 1990; Airs and Archer, 2010), and (iii) can be readily assimilated by marine bacteria (Kiene, 1998) as a carbon and N source (Kortstee, 1970; Kappes et al., 1999). Because choline contains nitrogen (N) (C:N = 5:1) it could alleviate N limitation of hydrocarbon degraders (Atlas and Bartha, 1998a,b). Furthermore, some choline derivatives, such as LCC, have properties of cationic surfactants (Totland and Nerdal, 2012) and their enzymatic hydrolysis produces choline and a fatty acid; both of which are naturally occurring compounds with high turnover rates in marine systems (Kortstee, 1970; Kiene, 1998; Mopper and Kieber, 1991). Our objectives were to determine the effectiveness of choline derivatives for bioremediation of moderately to extensively weathered hydrocarbons and to compare their efficiencies relative to inorganic N and phosphorous (P) in accelerating hydrocarbon degradation in sediments contaminated with crude oil.

2. Methods

2.1. Experimental setup

The biodegradation of crude oil was monitored in dark incubations maintained at 22 ± 2 °C in airtight 500 ml mesocosms, each containing 200 g of sediment (dry weight). Sediments were collected on October 6, 2011 from a beach at the North-East side of Dauphin Island, AL (30°15′04.11″N; 88°04′49.27″W) in the northern Gulf of Mexico. An aliquot of Louisiana sweet crude oil was mechanically weathered by placing it in an open container under a fume-hood, in the dark, on a mechanical shaker (85 rpm) for 5 days. The density, determined from the measured mass to volume ratio, of the resulting moderately weathered Louisiana crude oil was 0.90 g/cm^3 and sediments were contaminated with the weathered crude oil at a concentration of 4000 mg kg^{-1} sediment. Four treatments, with 5 replicates each, consisted of (i) sediments contaminated with oil; (ii) oil contaminated sediments amended with LCC; (iii) oil contaminated sediments amended with TCC; and (iv) oil contaminated sediments amended with inorganic N, P, and glucose (NPG).

LCC (TCI America), TCC (TCI America), or NPG (glucose, NH_4NO_3, and KH_2PO_4, Fisher Scientific) were added in the LCC, TCC, NPG treatments, respectively. Appropriate amounts were added in each of the treatments so that the increase in carbon content (335 mg C kg^{-1} sediment) to the sediments was equivalent to 10% of the carbon contained in the oil (3360 mg C kg^{-1} sediment) for each treatment. The varying C:N ratios in LCC and TCC resulted in higher N addition in the TCC (55.8 mg N kg^{-1} sediment) compared to the LCC (23.0 mg N/kg sediment) treatment. In the NPG treatment, 23.0 mg N kg^{-1} and 3.28 mg P kg^{-1} were added resulting in an N:P ratio of 7:1, similar to marine organic matter N:P ratios (Redfield, 1958). Parallel incubations were initiated for examining mineralization of indigenous carbon in the sediments as well as the mineralization of LCC, TCC, and NPG in the absence of oil. We here thereafter refer to these as pristine sediments and pristine LCC, TCC, or NPG treatments. These treatments, with triplicates, each were amended with LCC, TCC or NPG at quantities identical to those added to the oil contaminated sediments.

On day 7, one random replicate in the oil contaminated sediments and oil contaminated sediments amended with LCC, TCC, or NPG was removed for hydrocarbon analysis. On days 34 and 55, an aliquot of sediment from two randomly selected mesocosms was removed and analyzed for hydrocarbons. On day 34, following CO_2 concentration measurements, to the remaining replicates in each of the LCC, TCC, and NPG treatments (pristine and oil contaminated), second additions of LCC, TCC or NPG were made at quantities identical to the addition at the start of the experiment. This second addition on day 34 allowed us to further investigate mineralization and possible enhancement in biodegradation rates of the residual oil that was extensively weathered in response to additions of choline derivatives or NPG.

2.2. CO_2 concentration

Headspace CO_2 concentrations in each mesocosm were measured daily with a Shimadzu 8A gas chromatograph equipped with a thermal conductivity detector (GC/TCD). Following headspace analyses, mesocosms were re-aerated with air containing a known CO_2 concentration (380 ppm) for 2 min at a 0.5 L min^{-1} flow rate and headspace CO_2 concentrations were measured again and subtracted from the next day's headspace concentrations. The GC/TCD was calibrated with 0.0301%, 0.1%, 1%, 2.5%, and 5% CO_2 standards.

2.3. Sediment characteristics and hydrocarbon analyses

Physiochemical parameters including pH and salinity were measured in the field with a YSI (556 MPS) meter at the time of sediment collection. The gravimetric water content of the sediments was determined by oven drying of sediments for 48 h at 60 °C. Inorganic nutrients were extracted from sediment samples as previously described (Horel et al., 2012a,b) and analyzed for NO_2^-, NO_3^-, NH_4^+, and PO_4^{3-} with a SKALAR auto analyzer. Concentrations are reported as mg kg^{-1} dry weight sediment.

Hydrocarbon concentrations were determined with a Shimadzu gas chromatograph equipped with a flame ionization detector (GC/FID) after extraction according to EPA 3550 and AK 102 diesel range organics methods. In addition, gas chromatography with mass spectrometry (GC–MS Total Ion Chromatograph, Thermo Scientific Instruments, Trace Ultra GC Gas Chromatography coupled with Triple quadruple TSQ Quantum GC Mass Spectrometry) was used to determine concentrations of biomarkers (pristane and phytane). The concentration of total petroleum hydrocarbons in diesel and oil ranges C_{10}–C_{32} is reported in mg kg^{-1} dry weight sediments.

2.4. Microbial enumeration

The most probable number (MPN) technique was used to estimate the number of hydrocarbon degrading bacteria (polycyclic aromatic hydrocarbon [PAH] degraders, alkane, and total petroleum hydrocarbon (TPH) degraders) originally present in the soil. Sediment samples were prepared for microbial enumeration as described in Horel et al. (2012a). Briefly, triplicate sediment samples (1 g) were suspended in 10 ml 1% (w/v) sodium pyrophosphate ($Na_4P_2O_7$) with 2% NaCl. To

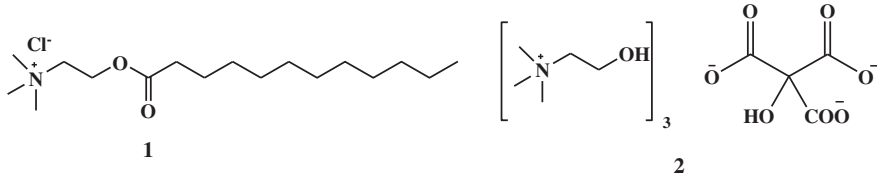

Fig. 1. Structure of lauroylcholine chloride (LCC) **1** and tricholine citrate (TCC) **2**.