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Biodegradation rate of diesel range *n*-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia*

Gita Mohanty, Suparna Mukherji*

Centre for Environmental Science and Engineering (CESE), Indian Institute of Technology (IIT) Bombay, Powai, Mumbai 400076, India

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Abstract

The biodegradation rate of hydrocarbons is an important consideration determining the time scale for bioremediation in oil-contaminated environments. Two naturally occurring bacterial cultures, *Exiguobacterium aurantiacum* and *Burkholderia cepacia*, were capable of utilizing diesel oil as the sole source of carbon and energy by induction of hydrophobic cell surfaces with water contact angle greater than 70°. The cultures demonstrated good degradation characteristics for diesel range *n*-alkanes (C9–C26) and were also capable of degrading pristane. A significant correlation was observed between maximum decay rate (MTR) of individual *n*-alkane peak area and initial abundance of *n*-alkanes in diesel ($r^2 = 0.79$ and 0.97 for *E. aurantiacum* and *B. cepacia*, respectively). Thus, MTR (day^{-1}) was essentially constant, in the range of 0.07–0.20, for *n*-alkanes with a wide range of carbon numbers from C12 to C26. Biodegradation altered the relative abundance of *n*-alkanes in diesel and resulted in a loss of symmetry in *n*-alkane distribution. C9, C17–C19, and C26 were completely degraded by both the cultures. In *B. cepacia*, the residual diesel was enriched in the higher carbon number *n*-alkanes C20–C25. The MTR of *n*-C16 present as a component of diesel was comparable to that for *n*-C16 when present as sole substrate for *B. cepacia* but not for *E. aurantiacum*.

Scientific relevance: Two cultures *E. aurantiacum* and *B. cepacia* were capable of utilizing diesel oil as sole substrate and exhibited uniform decay rates for a wide range of *n*-alkanes from C12 to C26. In contrast, most researchers have reported decreasing degradation rate with increasing carbon number.

Keywords: Oil; NAPL; Biodegradation; Bacteria; Hydrophobicity

1. Introduction

Biodegradation of petroleum hydrocarbons in oil and other non-aqueous phase liquids (NAPLs) by bacterial cultures has been reported in both batch and field scale studies (Mills et al., 1999; Olson et al., 1999; Gogoi et al., 2003; De Oteyza et al., 2004). The rate and extent of biodegradation in batch cultures was affected by NAPL composition and concentration, culture characteristics, concentration and their inherent NAPL uptake mechanism, nutrient media composition, and other cultivation conditions, such as temperature, pH, and speed of rotary shaking. Important variables affecting the success of bioremediation in contaminated sites were identified as: nature of the NAPL contaminants, duration of contamination, available or applied consortia and their predominance, nutrients applied, and other environmental parameters. NAPL biodegradation/bioremediation is usually a slow process due to the hydrophobic nature of the contaminants and consequent bioavailability limitations. Longer acclimatization time is required for microorganisms to degrade complex hydrocarbon substrates where multiple substrate interaction effects and interaction between cultures degrading the various fractions can potentially play a dominant role.

Various components in crude oil are metabolized simultaneously, but some are metabolized at a faster rate than others. Based on studies testing the effectiveness of products for bioaugmentation and biostimulation, Aldrett et al. (1997) reported on the significant difference in biodegradation rates of the saturates and aromatics in

*Corresponding author. Tel.: +91 22 2576 7854; fax: +91 22 2576 4650.
E-mail address: mitras@iitb.ac.in (S. Mukherji).

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crude oil. Components more resistant to degradation are degraded only after the more susceptible components present in the complex mixture are degraded. In complex NAPLs containing *n*-alkanes, the aerobic degradation of *n*-alkanes was found to be inversely related to the carbon chain length (Setti et al., 1995). Reduction in biodegradability with increasing *n*-alkane carbon number in motor oil was reported by Plohl et al. (2002) for a pure bacterial culture associated with cyanobacteria. Within 5 days, the extent of biodegradation for *n*-alkanes grouped as C15–C22, C22–C30, and C30–C40 were 70%, 45%, and 20%, respectively. Ward et al. (2003) reported maximum degradation rate (μg/h) inversely related to chain length for C8–C11, in crude oil for mixed and pure cultures. Using compound-specific isotopic fractionations for hydrogen and carbon for estimating biodegradation, Cao and Wang (2004) confirmed this observation for two pure strains of *Nocardia* sp. using a synthetic petroleum hydrocarbon mixture containing C10–C36 *n*-alkanes. Pristane (a highly branched alkane), which could also serve as a growth substrate when present as a pure component, could not be degraded as long as *n*-alkanes were present in the synthetic mixture. The components 5α-androstane (a cycloalkane), and hexamethylbenzene (an aromatic) in the synthetic mixture were resistant to biodegradation.

The present study focuses on the rate and extent of biodegradation of diesel range *n*-alkanes and the branched alkane pristane, by two naturally occurring bacterial cultures, *Exiguobacterium aurantiacum* NCDO 2321 (T) and *Burkholderia cepacia* LMG 12614t2, isolated from contaminated soil and sediments, respectively, using diesel as the sole source of carbon and energy. Both the cultures exhibited high cell surface hydrophobicity when grown on diesel, and were capable of direct interfacial uptake. The objective of this study was to determine the effect of carbon chain length, for *n*-alkanes in the range C9–C26, on the rate of *n*-alkane biodegradation by these cultures. A study was also conducted to compare the maximum decay rate of *n*-hexadecane when present as a sole substrate and as a component in diesel for the two cultures, *E. aurantiacum* and *B. cepacia*.

2. Materials and methods

Diesel oil obtained from a petrol pump (Mumbai, India) was stored in airtight containers and was used as received. All other inorganic mineral media components were of analytical reagent grade and were obtained from S.D. Fine-Chemicals (Mumbai, India). The 5α-androstane was obtained from Sigma-Aldrich.

2.1. Bacterial strains and culture conditions

The two cultures used for this work were Gram-positive *E. aurantiacum* NCDO 2321 (T) and Gram-negative *B. cepacia* LMG 12614t2. These cultures isolated from contaminated soil and sediments using diesel as the sole substrate were identified based on 16S rDNA analysis and depicted 99.3% and 99.6% match, respectively (Bangalore Genei, Bangalore, India). Both the cultures could utilize aliphatic hydrocarbons as the sole source of carbon and energy. They did not release extracellular biosurfactants/bioemulsifiers but exhibited high cell surface hydrophobicity when grown on *n*-hexadecane and diesel (Mohanty, 2006). The cell surface hydrophobicity could be established on the basis of contact angle measurement on cells deposited on a 0.45-μm cellulose acetate filter. Early stationary phase cells of *E. aurantiacum* and *B. cepacia* grown on diesel had a water contact angle of 82° and 76°, respectively, while for *n*-hexadecane-grown cells the water contact angles were 52° and 59°, respectively. In contrast, the dextrose-grown cells of both the cultures had a low water contact angle in the range of 29–31°.

Methods for NAPL (*n*-hexadecane and diesel) degradation studies and media used were reported by Mukherji et al. (2004). The studies were conducted in 500-mL conical flasks containing 100 mL of mineral media, 1 mL NAPL (*n*-hexadecane/diesel oil), and 1 mL inoculum from late log growth phase. Growth of the microorganisms was observed as an increase in absorbance (at 600 nm) of the culture broth over time, which was correlated to viable count for diesel degraders based on predetermined correlation curves. The viable count of diesel-grown cultures was determined using the five-tube MPN technique as reported by Biswas et al. (2005). The rate and extent of *n*-hexadecane/diesel range *n*-alkane biodegradation by *E. aurantiacum* and *B. cepacia* cultures were studied using multiple batch cultures over a period of 15 days. Controls devoid of inoculum were incorporated to indicate the natural weathering/abiotic losses. The cultures and controls were incubated in a rotary shaker set at 120 rpm and 30 °C. After extraction of residual NAPL, it was quantified gravimetrically and biodegradation of individual constituents in diesel was studied using gas chromatography (GC).

2.2. Extraction and analysis of residual diesel/*n*-hexadecane by GC

Extraction of residual *n*-hexadecane/diesel oil was performed as discussed by Mukherji et al. (2004) with some modifications; the biosolids were abiotically associated with oil and hence, centrifugation of biosolids and recovery of oil associated with the biomass pellet with solvent washing was incorporated. The aqueous phase was acidified and extracted thrice with hexane (extraction ratio 10:1). The combined solvent extract from the various stages was passed through anhydrous sodium sulfate (Na_2SO_4). A part of the extract was used to quantify the extracted diesel gravimetrically and another part was used for GC analysis.

The range of *n*-alkanes present in diesel and the most predominant *n*-alkanes were identified using a GC–MS (Agilent 5890), equipped with an HP1 column and using helium as carrier gas. Routine analysis of diesel was performed in the GC-FID (Agilent 6890), using the HP5 capillary column with 5% phenyl methyl siloxane as stationary phase and dimensions 30 m (*L*) × 0.32 mm (i.d.); film thickness was 0.25 μm. Nitrogen was used as a carrier gas, the injection volume was 2 μL, and a splitless injection mode was employed. The oven temperature programming was as follows: initial temperature 50 °C, with hold time 1 min; ramping at 10 °C/min up to 150 °C, with hold time 1 min; ramping at 5 °C/min up to 200 °C, with hold time 1 min; and the final ramping at 25 °C/min up to 280 °C, with hold time 15 min; total run time was 41.2 min. This temperature programming resulted in good peak resolution. The injector temperature was set at 200 °C while the detector temperature was set at 280 °C. Both qualitative and quantitative analysis was performed using an *n*-alkane reference calibration mixture (D-2887; Accu Standards Inc., New Haven, CT, USA) containing 17 *n*-alkanes over the range C6–C44. Calibration curves for individual *n*-alkanes were prepared from dilutions of the calibration mixture over the range of 0.01–0.5 mg/mL. For all standards and samples, 0.02 mg/mL of 5α-androstane was added as internal standard (IS) and a dilution factor (DF) of 10 was employed before injection in the GC. *n*-Hexadecane was analyzed under the same condition in the GC-FID after modifying the oven temperature program: initial temperature 100 °C, with hold time 1 min; ramping at 25 °C/min up to 250 °C, with hold time 3 min. The total run time was 10 min. The injector temperature was set at 100 °C while the detector temperature was set at 300 °C. For quantitative estimation of *n*-hexadecane, a standard curve was generated over the concentration range 5–20 and 5 μg/mL of *n*-octadecane was added as internal standard. The retention time and peak