

OPTIMAL NITRATE CONCENTRATION FOR THE BIODEGRADATION OF *n*-HEPTADECANE IN A VARIABLY-SATURATED SAND COLUMN

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ABSTRACT

Bioremediation of oil spills on beaches commonly involves the addition of nutrients (especially nitrogen and phosphorus) to stimulate the growth of indigenous oil-degrading bacteria. Very little information is available regarding the relationship between nutrient concentration and the rate of oil biodegradation. This information is necessary to design an appropriate nutrient delivery technology. We used continuous-flow beach microcosms containing heptadecane-coated sand (2.0 g per kg of dry sand) to evaluate the effect of nitrate concentration on the hydrocarbon biodegradation rate. Heptadecane biodegradation was determined by monitoring oxygen consumption and carbon dioxide production in the microcosms. The maximum biodegradation occurred at 2.5 mg nitrate-N l⁻¹. Nitrogen recycling by the biomass was evidenced by the presence of microbial activity at zero influent nitrate concentration.

Keywords: Bioremediation, oil, nutrient, beaches, shores.

INTRODUCTION

Accidental oil spills on beaches can constitute major environmental problems. One available alternative for clean-up involves stimulating indigenous microorganisms that metabolize the various constituents of the petroleum [1]. Microbial activity is enhanced by the addition of nutrients to the polluted area [2], especially when the background nutrients are at limiting concentrations.

The usual strategy for nutrient supplementation involves establishing favorable ratios of carbon to nitrogen, C/N, and carbon to phosphorus, C/P, or determining the nitrogen demand of the target oil [3]. These methods are based on the stoichiometry of oil biodegradation, i.e., determining the mass of nutrient that is required to degrade a given mass of oil. Oil-contaminated shorelines, however, are open environments where a new supply of water arrives with every tide. At the same time, dissolved nutrients that are added to the beach surface to stimulate oil biodegradation are washed out of the bioremediation zone by the periodic flushing effected by the tide and waves [4,5]. Therefore, successful bioremediation of coastal oil spills is dependent more on the kinetics of nutrient consumption than on

stoichiometry; that is, it is important to maintain a sufficient concentration of nutrients in contact with the oil to support the maximal oil biodegradation rate throughout the weeks or months that are required to complete the clean-up. Higher concentrations will provide no added benefit. Unfortunately, much less work has been done on determining the nutrient concentrations at which maximal oil biodegradation rates occur than on determining the stoichiometry of oil biodegradation. The purpose of this research was to determine the minimum nitrogen concentration needed for maximum hydrocarbon biodegradation in open microcosms that simulate a sandy beach at low tide. Investigation of the phosphorus effects is left for future work.

The experimental approach consisted of immobilizing the hydrocarbon substrate, *n*-heptadecane, on sand and monitoring its biodegradation in continuous-flow beach microcosms containing attached hydrocarbon-degrading microorganisms. The microcosms were connected to a respirometer. Each system was isolated from the atmosphere to ensure proper functioning of the respirometer, which provides oxygen gas whenever the pressure in the closed system drops due to oxygen consumption. The effect of nutrient concentration was determined by flushing the sand

columns continuously with a mineral salts solution containing nitrate at concentrations varying from 0 to 5 mg N l⁻¹. The rate of heptadecane biodegradation in each microcosm was monitored by continuously recording the amount of oxygen that was supplied to the systems by a respirometer. Inorganic carbon (i.e., CO₂) and nitrate concentrations in the reactor effluents were also monitored periodically to further characterize hydrocarbon biodegradation.

EXPERIMENTAL SET-UP

The effect of the concentration of nitrate on the rate of heptadecane biodegradation was studied using six identical beach microcosms, each receiving different influent concentrations of nitrate. A schematic of the beach microcosms is shown in Figure 1. Each reactor consisted of two primary vessels: a sand column and a nutrient reservoir bottle. A peristaltic pump drew water from the bottom of the 21 l reservoir and pumped it to the top of the reactor. Effluent from the microcosms returned to the top of the reservoir bottle. The head spaces of the two vessels and the air volume below the sand column screen were connected via flexible tubing. Oxygen, which was supplied

by a respirometer (N-Con Systems, Larchmont, NY), entered near the top of the reactor column.

The sand columns

The sand columns consisted of glass tubes 7.6 cm (ID) by 38.7 cm long that contained 1.0 kg of sand. A screen placed 5.7 cm from the bottom of the reactor served to retain the beach sand while allowing continuous flow of a nutrient-amended mineral salts solution. The average dimension of the screen openings was 112 μm. A 3 cm layer of glass beads was placed above the sand to distribute the influent flow evenly across the sand surface matrix.

The sand had a very narrow grain-size distribution varying from 0.6 mm to 0.85 mm. The bulk (dry) density and the porosity of the sand were measured and found to be 1.43 kg l⁻¹ and 0.38, respectively. The unsaturated hydraulic characteristics of the sand were estimated by fitting the van Genuchten model [6] to the experimental capillary-retention curve [7] obtained from the sand. The van Genuchten parameters were estimated at $a = 0.1 \text{ cm}^{-1}$ and $n = 7.1$. The inverse of a provides an estimate of the capillary fringe (zone of considerable moisture) above the water table. The capillary fringe, in this case, is about 10 cm. The term n is the coefficient of uniformity; it increases with increasing uniformity of the pores. The estimated value ($n = 7.1$) falls in the high end of the value range reported in the literature [8], which is expected for sands with narrow grain-size distributions. The residual saturation ratio of the van Genuchten model, which is the saturation ratio (soil moisture divided by porosity) at a very low negative water pressure, was set at 0.05, which is a reasonable value for this type of sand [6].

Tracer study

Prior to the start of this experiment, a tracer study was performed to determine whether short-circuiting occurred between the influent and effluent flows of the reservoirs. One 21 l reservoir was isolated and filled with 3.5 mg nitrate-N l⁻¹ solution. One pump was used to draw water out of the reservoir while another pumped water into the reservoir. The two pumps were operated at the same flow rates used in the microcosm experiment. The feed to the reservoir was free of any nitrate and the effluent from the reservoir was monitored for nitrate-N. The tracer experiment was replicated, and the concentration of nitrate-N in the effluent from both experiments is shown in Figure 2. The data in Figure 2 suggest that very little mixing occurred within the reservoir during the first 48 hours, which corresponds to the service period of each batch of reactor feed. For the sake of providing a reference for comparison, the effluent concentration of nitrate-N from the reservoir that would have resulted had the reservoir been completely mixed is also provided in Figure 2.

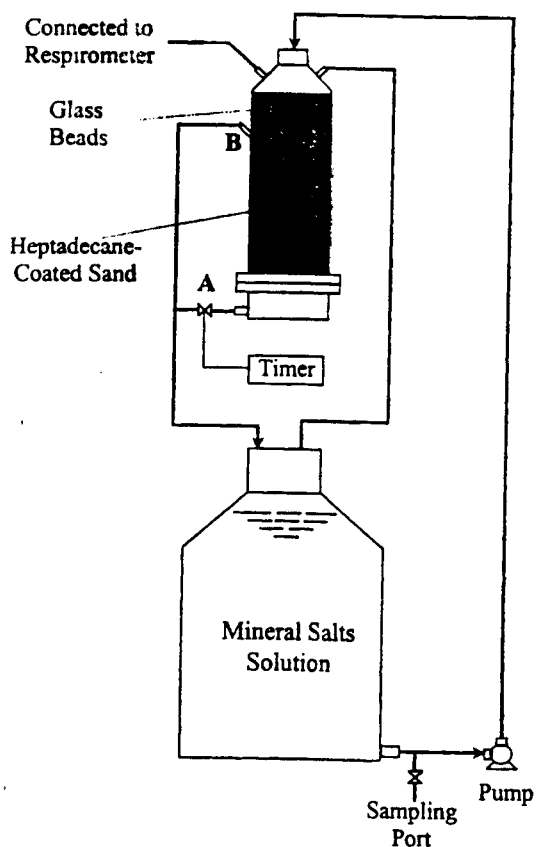


Figure 1. Schematic diagram of a microcosm for n-heptadecane biodegradation.

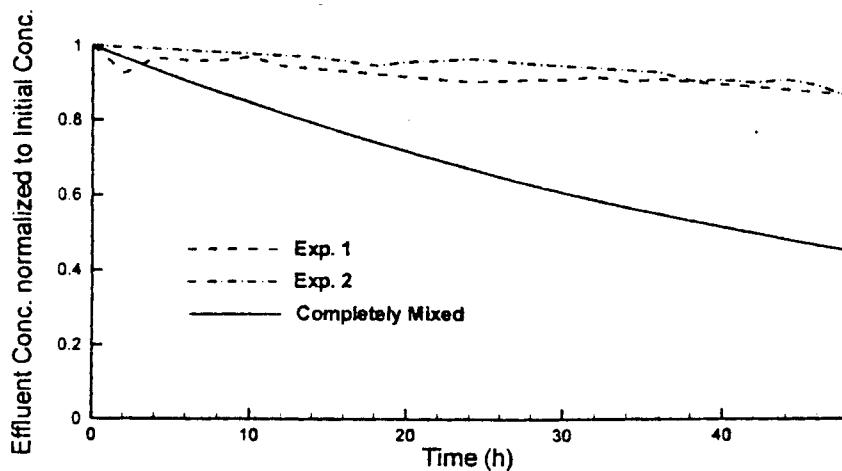


Figure 2. Comparison between effluent concentration of duplicate tracer studies in the 21 l reservoir, and that obtained by assuming that the reservoir is completely mixed.

Heptadecane loading

Prior to being placed in the reactor, the sand was loaded at room temperature (about 25 °C) with 2 000 mg of heptadecane. At this temperature heptadecane, which has a melting point of 20 °C, is a liquid. The sand was loaded manually by pipetting heptadecane into 1 000 ml Erlenmeyer flasks each containing 1.0 kg of sand. The flasks were sealed with rubber stoppers and shaken by hand for 5 minutes; the length of stroke was about 30 cm and the frequency was about 3 cycles per second. The uniformity of mixing was determined by visual inspection and verified by extracting several small samples removed from various locations throughout the sand volume. The heptadecane-coated sand was then put in the glass column and the whole system was cooled to 18 °C, which caused the heptadecane to solidify onto the sand particles.

Average sand moisture

The flow rate through the reactors was 300 ml h⁻¹, resulting in a percolation (flux) rate of 4.05 cm h⁻¹ in the sand columns. Percolation rates in natural beaches vary widely, depending on factors such as the position relative to the swash zone (uprush-backwash of waves), the wave size, and the characteristics of the substratum. Riedl [9] reported values up to 40 cm h⁻¹ for exposed beaches, but the flux rates on sheltered beaches could be as much as three orders of magnitude lower. Therefore, the flux rate used in this study was near the high end of the natural range. Because a coarse sand was used, the flux (4.05 cm h⁻¹) was lower than the saturated hydraulic conductivity of the sand, and unsaturated-flow conditions were observed (visually) in about the top two-thirds of these sand columns. The screen holding the sand in each reactor formed a capillary barrier

[10], causing the sand at the bottom of the columns to be essentially under saturated flow conditions. A capillary barrier occurs whenever a fine layer overlays a coarse layer and the flux rate is lower than the saturated hydraulic conductivity of the fine layer. A pressure build-up above the interface is needed to allow the water to leave the finer medium. In this study, the air volume below the screen can be thought of as a porous medium with a very high hydraulic conductivity. Theoretically, water does not leave the sand if its pressure at the screen is below atmospheric pressure. However, imperfections at the screen-sand and the screen-glass contacts permit the water to leave even though the average pressure over the cross section is below atmospheric. At the same time, the incoming water increases the average soil moisture in the column. Because the water pressure was not measured in this study, the pressure at the screen was assumed equal to atmospheric pressure. The soil moisture distribution with elevation was computed from the van Genuchten model using the numerical model of Boufadel *et al.* [11]. The average soil moisture ratio, which nominally varies between 0.05 and 1.0, was computed to be 0.65 in the sand column.

Nutrients solution

The influent to all of the microcosms consisted of an aqueous solution of nutrients and micronutrients in deionized water. The concentrations of the various nutrients are listed in Table 1. Because the focus of this work was to investigate the effects of nitrate on heptadecane biodegradation, all nutrients other than nitrate were present at concentrations that were sufficient to ensure that they did not limit microbial growth. Influent nitrate concentrations of 0.0, 0.1, 0.5, 1.0, 2.5, and 5.0 mg nitrate-N l⁻¹ were provided to each microcosm.

Table 1. Inorganic nutrients in the influent solution

| Chemical | Concentration, mg l ⁻¹ |
|--|-----------------------------------|
| FeCl ₃ *6H ₂ O | 10 |
| NaCl | 125 |
| CaCl ₂ *2H ₂ O | 50 |
| Na ₅ P ₃ O ₁₀ | 600 |
| MgSO ₄ | 200 |
| MnSO ₄ | 0.0302 |
| ZnSO ₄ | 0.0428 |
| H ₃ BO ₃ | 0.0458 |
| Na ₂ MoO ₄ | 0.0404 |

The Biomass

A mixed culture of oil-degrading microorganisms, originally isolated from Slaughter Beach, Delaware in 1994, was grown in Bushnell-Haas medium [12] supplemented with 2% NaCl for three weeks using artificially weathered Alaskan North Slope crude oil (ANS521) as the growth-supporting substrate. Weathering was accomplished by nitrogen blow-down for 2 days under a hood. The culture was centrifuged and washed twice with sterile 2% NaCl to remove spent medium and any residual oil. The washed cells were resuspended in sterile 2% NaCl at about 10% of the original volume. The suspension (4.5 ml) was distributed into tubes containing 0.5 ml of glycerol, and the mixture was stored frozen at -70°C. The frozen cultures were thawed, centrifuged, and washed with sterile 2% NaCl to remove lysed cell debris, then resuspended and used to inoculate the reactors. The washed and resuspended contents of five frozen stock cultures were added to each microcosm at the start of each experiment. The initial biomass concentration was determined by analysis of lipid phosphate [13].

CHEMICAL ANALYSIS

Biodegradation of heptadecane in the beach microcosm reactors was continuously monitored by respirometry. The headspace in each microcosm was connected to a respirometer that delivered and recorded oxygen as it was needed to replace that which was consumed by oxidation of the heptadecane (Figure 1). Although oxygen was supplied continuously upon demand, the amount that was supplied was recorded by the computer at 2 h intervals. Dissolved oxygen (DO) in the 21 l reservoirs was checked each time they were changed, and it was the same as the influent DO (i.e. 8 mg l⁻¹).

Carbon dioxide that was produced by mineralization of the heptadecane was dissolved in the aqueous phase and, therefore, was continuously removed from the reactors in the effluent. The amount of CO₂ produced was determined in the nutrient reservoir at the beginning and end of each 2-day cycle by measuring the inorganic carbon concentration in the

medium using an organic carbon analyzer (Shimadzu TOC 5000; Shimadzu Corp., Tokyo, Japan). The difference in concentration was multiplied by the reservoir volume to obtain the mass of CO₂ that was produced.

Nitrate consumption was monitored in a similar manner to the method used for carbon dioxide: one sample was taken at the beginning and another at the end of each 2 day cycle. Nitrate was quantified by measuring the absorbance at 220 nm [14]. The nitrate concentration was corrected for interference from dissolved organic compounds by measuring the absorbance at 275 nm.

The amount of n-heptadecane remaining at the end of the experiment was determined by extracting the sand with hexane/acetone (50/50) and analyzing the extract using a Hewlett Packard 5890 series II gas chromatograph (GC) equipped with a flame ionization detector (FID) and an HP 7673 Auto Sampler. The sand was removed from the reactors in five equal vertical layers approximately 3.5 cm thick, which were each mixed well and preserved at 4°C. Sub-samples were then extracted and analyzed for n-heptadecane. Separate sub-samples were taken for moisture determination so that the concentration could be calculated on a dry weight basis.

The concentration of the initial active biomass was quantified by analysis of lipid phosphate [13]. This method assumes that the phospholipids measured came only from the cell membranes of living microorganisms (because phospholipids degrade rapidly upon cell death) and that the amount of lipid phosphate is related to the active biomass by a constant factor. In this study, a conversion factor of 1.92 g C/(nmol lipid phosphate) was used [13].

RESULTS

The initial biomass added to each microcosm was computed to be 7.5 mg. The longitudinal distribution of the heptadecane in each column at the end of the experiment was obtained by sampling from 5 layers of equal thicknesses.

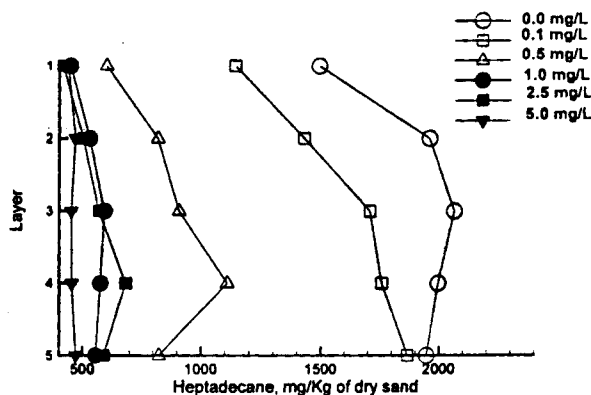


Figure 3. Heptadecane remaining in the sand columns corresponding to various influent nitrate-N concentrations. Layer 1 is the top layer.

