ANAEROBIC BIODEGRADATION OF LINEAR ALKYLBENZENE

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ABSTRACT

Linear alkylbenzenes (LAB) are commonly found in the environment due to their use as a precursor in the manufacture of linear alkylbenzene sulphonate (LAS) detergents, in which they remain as trace contaminants. Other uses include insulating oils in buried electricity transmission cables, from where they may enter soil and groundwater in the event of damage to the cable casing or joints. They are readily biodegraded under aerobic conditions but may be released into anoxic environments where they can persist for some time. Laboratory experiments show that LAB can be degraded under nitrate-reducing conditions, and isomeric analysis of the remaining LAB is good indicator of the degree of degradation. Work is continuing to identify changes in microbial diversity using denaturing gradient gel electrophoresis of rDNA polymerase chain reaction (PCR) products.

1 INTRODUCTION

Linear alkylbenzenes (LAB) have been produced commercially since the early 1960s for use in a variety of industries. The synthetic production pathway results in a mixture of isomers with a variety of alkyl chain lengths, dependent on the feedstock, with the phenyl group in any but the terminal position [22]. Conventionally, these isomers are described using the form nC_m LAB, where n = position of the benzene ring and m = number of carbon atoms in the longest aliphatic chain. For instance, using this convention, (1-methyl, undecyl)-benzene (also known as 2-phenyldodecane or dodecyl-2-benzene) is $2C_{12}$ LAB. Mixtures with an alkyl chain length of $C_{10\text{-}13}$ are used to insulate buried electrical transmission cables. Leakage of this cable oil may occur due to deterioration of cable components or through mechanical damage. When a leak is detected, contaminated soil is excavated to effect a repair to the cable, but oil that has migrated away from the immediate vicinity of the cable may be inaccessible, e.g. due to surface infrastructure or other buried services. Monitored natural attenuation is an attractive, and perhaps the only financially viable, strategy in such circumstances. Similar mixtures of $C_{10\text{-}13/14}$ LAB are also used in the manufacture of linear alkylbenzenesulfonate (LAS) detergents. Trace amounts remain in the detergent and thus are found in aquatic sediments where LAS contamination occurs. For the same reason, LAB is also seen in sewage sludge used to amend soils [17]. LAB was used for a period in the early 1990s as a major component of drilling oil [16; 21], and has also been identified as occurring naturally in the *n*-hexane fraction of some crude oils [4; 8]. Alkylbenzenes were reported in sewage extracts as early as 1974 [18], and LAB was identified as an environmental contaminant by Crisp *et al.* [2], who suggested that they may be derived from LAS detergents, either as unreacted feedstock or by microbial desulfonation. Eganhouse *et al.* identified LAB in marine sediments [6]. This was supported by Ishiwatari *et al.* [13], who found them associated with LAS detergent contamination in sediments in Tokyo Bay. However, LAB has not previously been studied as a bulk contaminant in its own right.

The relative amounts of different homologues of LAB in environmental samples can be used as an indicator of biodegradation. A commonly used predictor is the ratio of internal (I) to external (E) isomers of C_{12} LAB [24; 25], calculated as I:E = ($6C_{12}$ LAB + $5C_{12}$ LAB)/($4C_{12}$ LAB + $3C_{12}$ LAB + $2C_{12}$ LAB). It has been assumed that significant degradation only occurs under aerobic conditions [11]. However, indications that anaerobic degradation of LAB may occur [7; 14] have prompted investigations of cable oil biodegradation where the LAB is exposed to a variety of terminal electron acceptors (TEAs). Data from aqueous cultures of soil micro-organisms containing LAB cable oil as the sole carbon and energy source with a variety of electron acceptors is presented, and the linear regressions are compared using statistical methods.

2 MATERIALS AND METHODS

2.1 Batch cultures

Aqueous cultures consisting of universal bottles containing 20 ml of Bushnell-Haas (B-H) broth [1] with 100 µl LAB cable oil as the sole carbon source were inoculated with 100 µl of a suspension of organisms cultured from

cable oil-contaminated soil [14]. Aerobic (AER) and control (CON) bottles were incubated in a shaker/incubator at 25°C. Other cultures containing either no additional TEA (MET), nitrate (NIT), sulfate (SUL) or both nitrate and sulfate (ANA) were loosely capped and incubated at 25°C under an 85% N_2/10% CO_2/5% H_2 atmosphere. Added TEAs were calculated to be stoichiometrically 3-4 times more than that required for complete oxidation of the hydrocarbon. Sodium molybdate (1.0×10^{-2} mol l^{-1}) was added to the NIT cultures to inhibit sulfate-reduction [19]. Similarly, 1.0×10^{-2} mol l^{-1} sodium chlorate was added to inhibit nitrate reduction [12] in the SUL cultures. The NIT and SUL cultures contained 2.5×10^{-2} mol l^{-1} 2-bromoethanesulfonic acid (BES) to inhibit methanogenesis [15]. The CON bottles contained both additional nitrate and sulphate, but no inhibitors.

Three vials from each treatment were harvested and destructively analyzed at ten points over a period of 97 days. The contents of each universal bottle were poured into a 100 ml conical flask and LAB was extracted by shaking vigorously for 20 min in two volumes of *n*-hexane with 5 µl of pure $1C_{12}$ LAB a terminal isomer (and therefore absent from the synthetic mixture) as an internal GC standard to allow the peak areas to be quantified. A 2 ml aliquot of the organic fraction was transferred to a GC vial and analyzed by GC-MS:

2.2 GC-MS.

Separation was carried out on a Hewlett-Packard (Agilent) HP5890 Series II gas chromatograph fitted with an HP6890 autoinjector. The samples were applied as 1 µl splitless injections at 250°C to an HP5 (5% polysiloxane), 0.32 mm i.d., 0.25 mm film thickness, 30 m capillary column with He as the carrier gas. The column was held at 50°C for 2 min, increased at 10°C min^{-1}, held at 100°C for 2 min, increased at 2.5°C min^{-1}, held at 150°C for 2 min, increased at 5°C min^{-1}, and finally held at 300°C for 1 min. The GC effluent was fed to an HP5972 mass-selective detector at 280°C with m/z range of 35-500 scanned at ~1 s^{-1}. Peaks in the total ion current (TIC) chromatograms of fresh LAB and control bottle extracts were identified as being identical by searching against the Wiley138 mass spectrum library, and by examining the mass spectra directly. Peaks in the experimental bottles were identified by comparison to the control bottle chromatograms, with particular attention to retention times. The volume of LAB recovered was calculated by summing the TIC peak areas due to LAB isomers and comparing to the 5 µl $1C_{12}$ standard. The equation used was: cable oil volume = Σ(cable oil peak area)/$1C_{12}$ peak area $\times$ $1C_{12}$ volume.

Data from the experimental bottles were compared to the control bottles to discount removal of LAB via physical and chemical routes (sorption, volatilization, etc.). Loss from the control (CON) bottles, which were open to the atmosphere, was higher than anticipated, rendering them unsuitable for comparing to the sealed, anaerobic treatments. Anaerobic bottles (MET) containing no additional TEA, which were included to investigate whether methanogenic biodegradation occurred, showed no appreciable biodegradation and so were used as anaerobic controls.

3 RESULTS AND DISCUSSION

A variety of analytical methods have been used to analyze LAB degradation [5; 10; 23]. GC-MS has proved particularly sensitive [10] and the efficiency of the method is not heavily dependent on the details of extraction and GC methods. For soil, shaking in hexane has been shown to be up to 90% efficient, in terms of LAB extraction, over a range of soil types and moisture contents [9; 20]. In any event, because a ratio is being measured, rather than an absolute amount, the extraction efficiency is not critical, so long as the extraction method is not selective for different isomers. Extraction efficiency, being the volume of LAB recovered from the anaerobic control (MET) bottles divided by the volume of LAB added at the start of the experiment, was 74% (n = 33, standard deviation = 12.3%).

Many of the anaerobic treatments failed to show any degradation at all. Microbial growth was not quantified, but where it occurred it was visible as a pellicle at the LAB:water interface. No growth was observed in any of the bottles containing sulfate as the sole additional TEA. This confirms that the activity in the ANA bottles was due to nitrate-, rather than sulfate-reduction and is supported by the depletion of total oxidised nitrogen compared to the CON bottle in both NIT and ANA cultures (data not shown). This was to be expected since the original enrichments also failed to display measurable activity under these conditions and the inoculum was prepared using nitrate-reducing enrichments.

Anaerobic biodegradation was associated with dissimilatory nitrate reduction, with no degradation seen under sulfate-reducing or methanogenic conditions. The ratio of internal to external C_{12} LAB isomers was calculated and the $\log_{10}$ of this value regressed against % biodegradation of LAB cable oil for each of the experimental conditions. Since the intention was to detect any relationship between isomeric composition and degree of degradation, data points from anaerobic bottles with less than 10% degradation or a $\log_{10}$ (I:E) of less than 0.02 were discarded. Data from Takada and Ishiwatari [24] were similarly analyzed. The linear regression data have similar slopes and intercepts and are summarized in Table 1.

Table 1. Linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (I:E) nC_{12} LAB isomer ratio with different terminal electron acceptors

Terminal electron acceptor	Slope	Intercept	R^2 (%)
Aerobic (O_2)	73.0	17.4	61.0
Multiple TEAs (anaerobic – nitrate and sulphate)	92.1	13.0	97.6
Nitrate (plus molybdate to inhibit sulphate reduction)	126.9	13.4	79.0
Aerobic (O_2) data from Takada and Ishiwatari [24]	81.0	15.0	96.0
Composite regression using all data	77.9	16.4	84.3

The linear fit obtained using all the data was found to be significant ($p < 0.001$). The regression lines for aerobic and anaerobic biodegradation were checked to see whether they were statistically different by comparing them to this regression as described by Draper and Smith [3], and the null hypothesis - that the slopes and intercepts of the individual regressions were identical to the composite line - was accepted in every case ($p < 0.05$). All the available degradation data were plotted (Figure 1) to yield a regression equation that may be applied to field data where the redox history is unknown.

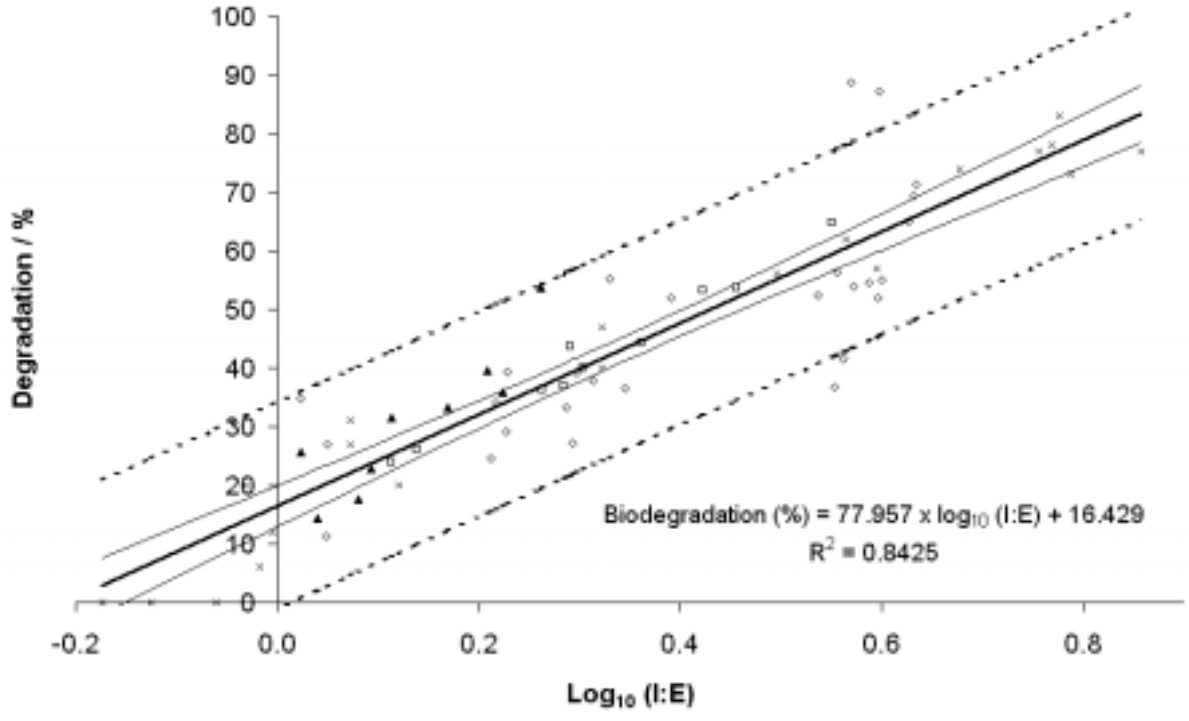

Figure 1. Plot of % biodegradation of LAB vs. $\log_{10}$ internal:external (I:E) nC_{12} LAB isomer ratio with linear regression (heavy) line, 95% confidence (light) and 95 % prediction intervals (broken lines). Data points are from individual bottles: aerobic (◊), anaerobic containing multiple TEAs (□) or nitrate alone (▲) and aerobic data from Takada & Ishiwatari [24](×)

LAB is biodegraded in liquid culture under both aerobic and nitrate-reducing conditions. That the aerobic degradation was so rapid using anaerobically cultured inoculum suggests that facultative anaerobes may play a significant role. Differential degradation of internal and external isomers follows similar patterns under a range of

conditions, while physical and chemical removal does not significantly favor any isomer. The ratio of internal to external nC_{12} isomers of LAB in the *n*-hexane fraction may provide a robust indicator of the degree of biological degradation in soils that have been contaminated with LAB, even where the redox history of the site is unknown.

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