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Anaerobic biodegradation of linear alkylbenzene: isomeric ratio as a monitoring tool

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Abstract

Linear alkylbenzenes (LABs) are common environmental contaminants associated with a range of industrial and domestic activities. Although natural environments receiving LABs may exhibit a range of redox conditions, until now only aerobic biodegradation of these compounds has been demonstrated. Where LAB contamination occurs, it is important to identify degradation and measure its extent in order to make decisions on whether monitored natural attenuation (intrinsic bioremediation) is sufficient, or whether active remediation techniques are required. We have demonstrated that LABs are degraded under both aerobic and nitrate-reducing conditions. Statistical analysis of these results and published data indicate that the C_{12} LAB isomeric ratio varies with biodegradation, independently of the terminal electron acceptor used. Biodegradation (B, %) can be estimated from the ratio of internal to external isomers of C_{12} LAB by the equation $B = 78 \times \log_{10} (I{:}E) + 16.4$. This relationship can be used to determine the degree of biodegradation of LABs in a range of environments, including sites where the redox history is unknown, making it a powerful yet simple tool for monitoring LAB biodegradation in the environment.

Key words: aerobic, bioremediation, LAB, natural attenuation

INTRODUCTION

Linear alkylbenzenes 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 (Swisher *et al.* 1961). 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 (2-phenyldodecane or dodecyl-2-benzene) is $2C_{12}$ LAB.

LAB mixtures with alkyl chain lengths of C_{10-13} 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 repair 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 strategy in such circumstances.

Similar mixtures of $C_{10-13/14}$ LAB are also used in the manufacture of linear alkylbenzenesulphonate (LAS) detergents. As a result of incomplete sulphonation of the raw LAB, trace amounts remain in the deter-

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gent and thus are found in aquatic sediments where LAS contamination occurs (Ishiwatari *et al*. 1983). LABs were first reported as environmental contaminants by Ishiwatari *et al.* (1983), who found them associated with LAS detergent contamination in sediments in Tokyo Bay. For the same reason, LABs are also seen in sewage sludge used to amend soils (Mangas *et al*. 1998). LAB was used for a period in the early 1990s as a major component of drilling oil (Steber and Herold 1995; M-I. $_{L.L.C}$ 1998) and they have been identified as occurring naturally in the *n*-hexane fraction of some crude oils (Ellis *et al*. 1996; Dutta and Harayama 2001). Frysinger *et al.* (2003) were able to use two-dimensional gas chromatography (GC–GC) to identify LAB in the previously unresolved complex material extracted from a petroleum hydrocarbon-contaminated sediment. However, LABs have not previously been studied as bulk contaminants in their own right.

It has been shown that internal (phenyl position > 4) isomers of LAB are more resistant to microbial degradation than external (phenyl position ≤ 4) ones (Bayona *et al.* 1986). This preferential degradation has been demonstrated both in the field and using defined bacterial cultures including a *Pseudomonas* sp. (Bayona *et al.* 1986) and *Nocardia amarae* (Bhatia and Singh 1996). Aerobic degradation mechanisms suggested by Bhatia and Singh (1996) all start with an ω-oxidation of a terminal methyl group, followed by β-oxidation of the alkyl chain. This is in agreement with the pathways seen elsewhere (Smith and Ratledge 1989; Smith 1990) for the degradation of terminal LAB isomers, where initial attack was at the methyl terminus of longer chains, but on the phenyl group if the chain was shorter. It is likely that steric hindrance by the delocalized π-electrons of the phenyl group contributes to the differential degradation of internal and external isomers.

The relative amounts of different homologues of LAB in environmental samples have been used as an indicator of biodegradation. One such predictor is the ratio of internal (*I*) to external (*E*) isomers of C_{12} LAB in aquatic sediments (Takada and Ishiwatari 1990; 1991). It has been assumed that significant degradation only occurs under aerobic conditions (Holt and Bernstein 1992). However, indications that anaerobic degradation of LAB may occur (Johnson *et al.* 2001) have led to investigations of cable-oil biodegradation where the LAB is exposed to a variety of terminal electron acceptors (TEAs). We present data from aqueous cultures containing LAB cable oil as the sole carbon and energy source with a variety of electron acceptors.

METHODS

Batch cultures

Aqueous cultures containing Bushnell–Haas medium with LAB as the sole carbon source were inoculated with organisms cultured from cable-oil-contaminated soil (Johnson *et al.* 2001). Aerobic and killed control flasks were incubated aerobically in a shaker/incubator. Other cultures containing either no additional TEA (MET), nitrate (NIT), sulphate (SUL) or both nitrate and sulphate (ANA) were incubated anaerobically under an 85% N_2/10% CO_2/5% H_2 atmosphere. Added TEAs were calculated to be stoichiometrically three to four times more than that required for complete oxidation of the hydrocarbon. Molybdate or chlorate was added to inhibit, respectively, sulphate or nitrate reduction (Newport and Nedwell 1988).

Flasks from each treatment were harvested and analysed at intervals. Immediately prior to the extraction step, 5 μL of $1C_{12}$ LAB (a terminal isomer, absent from the synthetic mixture) were added to serve as an internal GC standard to allow the peak areas to be quantified. The LAB was extracted by shaking samples for 20 min in *n*-hexane and the organic fraction analysed by GC–MS.

GC–MS

Separation was carried out on an HP5890 Series II gas chromatograph. 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 oven 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 flask extracts were identified as being identical by searching against the Wiley 138 mass spectrum library, and by examining the mass spectra directly. Peaks in the experimental flasks were

identified by comparison with the control flask chromatograms, with particular attention to retention times.

The removal rates from the experimental flasks were compared with those from the control flasks, to account for removal of LAB via physical and chemical routes (sorption, volatilization, etc.). Losses through volatilization in the control flasks were higher than anticipated, and so anaerobic flasks containing no additional TEA, which showed no appreciable biodegradation, were used as anaerobic controls. Since the intention was to detect any relationship between isomeric composition and degree of degradation, data points from anaerobic flasks with less than 10% degradation or a $\log_{10}$ (I:E) of less than 0.02 were discarded.

RESULTS AND DISCUSSION

A variety of analytical methods have been used to analyse LAB degradation (Takada and Ishiwatari 1985; Eganhouse 1986). Gas chromatography–mass spectrometry (GC–MS) has proved particularly sensitive and details of extraction and GC methods are not critical (Hartmann *et al*. 2000). 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 (Schwab *et al*. 1999; Fu *et al*. 2000). 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.

Many of the anaerobic treatments failed to show any degradation at all. No growth was observed in any of the flasks containing sulphate as the sole TEA. This confirms that the activity in the anaerobic flasks was due to nitrate-, rather than sulphate-reduction. This may have been due to the inoculum size or a lack of microorganisms capable of using a particular TEA with LAB as the sole carbon and energy source. The relatively small amount of biodegradation seen in the nitrate-containing flasks can be explained by the evolution of nitrite in these flasks (Johnson 2003). It seems likely that molybdate, added to the NIT flasks to inhibit sulphate reduction, also inhibited the reduction of nitrite (NO_2) to NO and N_2, thus hindering the reaction kinetics, though this has not previously been reported in the literature. This is unlikely to be a competitive inhibition, since the NO_3^- ion differs in both shape and charge from MoO_4^{2-} and SO_4^{2-}. One of the cofactors of nitrate reductase contains molybdenum, but it is not clear whether molybdate in solution affects the overall function of the enzyme system. The fact that LAB was degraded without complete reduction of nitrate to nitrogen gas suggests that biodegradation is coupled to the reduction of nitrate to nitrite. Burland and Edwards (1999) made a similar observation regarding the biodegradation of benzene.

Anaerobic biodegradation was associated with dissimilatory nitrate reduction, with no degradation seen under sulphate-reducing or methanogenic conditions. The ratio of internal to external C_{12} LAB isomers was calculated and the base-10 logarithm of this value regressed against the proportion of biodegradation (expressed as a percentage) of LAB cable oil for each of the experimental conditions. Data from Takada and Ishiwatari (1990) were similarly analysed. The linear regression data are shown in Figures 1–5 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 (%)
Oxygen (AER)	73.0	17.4	61.0
Nitrate and sulphate (ANA)	92.1	13.0	97.8
Nitrate (NIT)	127.0	13.4	79.0
Sulphate (SUL)	No degradation observed		
Aerobic data from Takada and Ishiwatari (1990)	81.4	14.5	96.4
Composite regression using all available data	78.0	16.4	84.3

There are several advantages to restricting the measure of isomeric composition to a single chain length. As chain length increases, so do the number of internal isomers. Since the number of external isomers is fixed at three over the range of chain lengths studied, different chain lengths cannot be compared directly. C_{12} LAB isomers are present as major components in currently available commercial LAB mixtures ($C_{10–14}$ and $C_{10–13}$). There is also a potential disadvantage to using a ratio; if all the external isomers are depleted, the ratio will tend to infinity. However, the inclusion of $4C_{12}$ as an external isomer means that the C_{12} I:E ratio in fresh LAB is close to unity and large values of the I:E ratio will only be approached at very high degrees of degra-

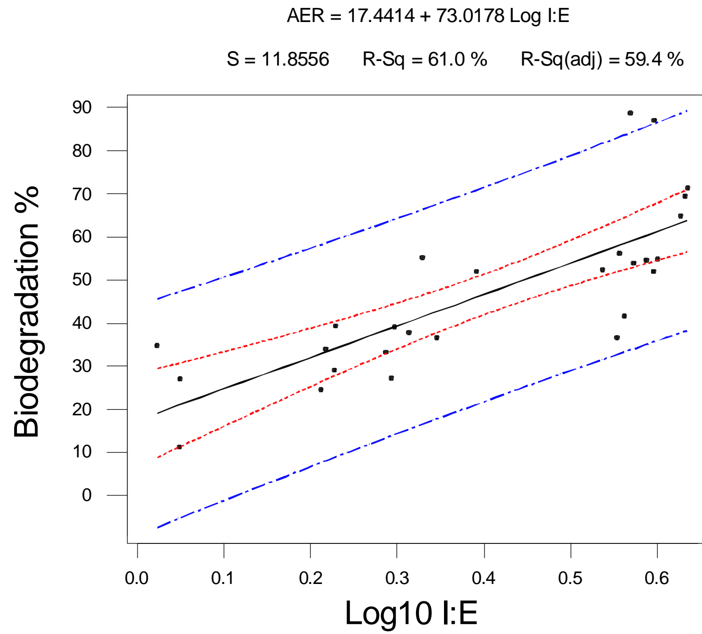

Figure 1. Linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (*I*:*E*) nC_{12} LAB isomer ratio under aerobic conditions, showing 95% confidence (- - -) and 95% prediction (- — -) intervals

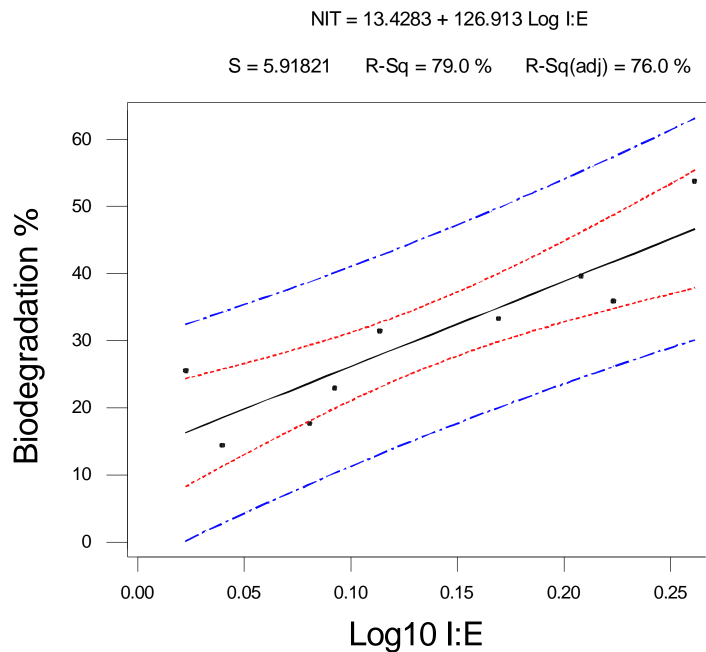

Figure 2. Linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (*I*:*E*) nC_{12} LAB isomer ratio under nitrate-reducing conditions, showing 95% confidence (- - -) and 95% prediction (- — -) intervals

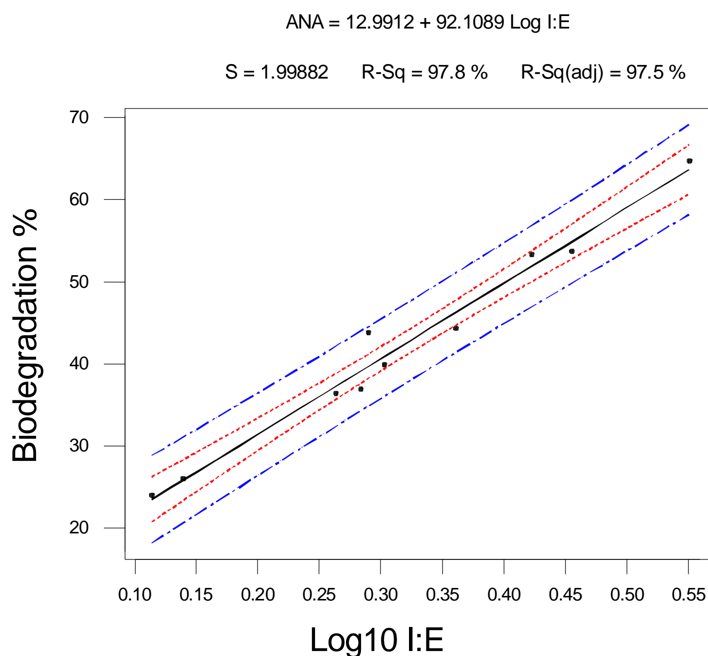

Figure 3. Linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (*I*:*E*) nC_{12} LAB isomer ratio under anaerobic conditions with multiple TEAs, showing 95% confidence (- - -) and 95% prediction (- — -) intervals

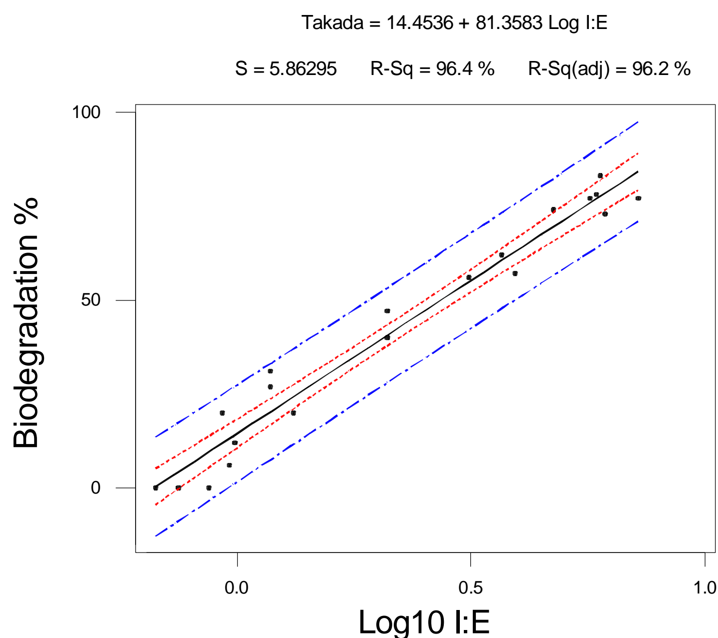

Figure 4. Linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (*I*:*E*) nC_{12} LAB isomer ratio under aerobic conditions: data from Takada and Ishiwatari (1990), showing 95% confidence (- - -) and 95% prediction (- — -) intervals.

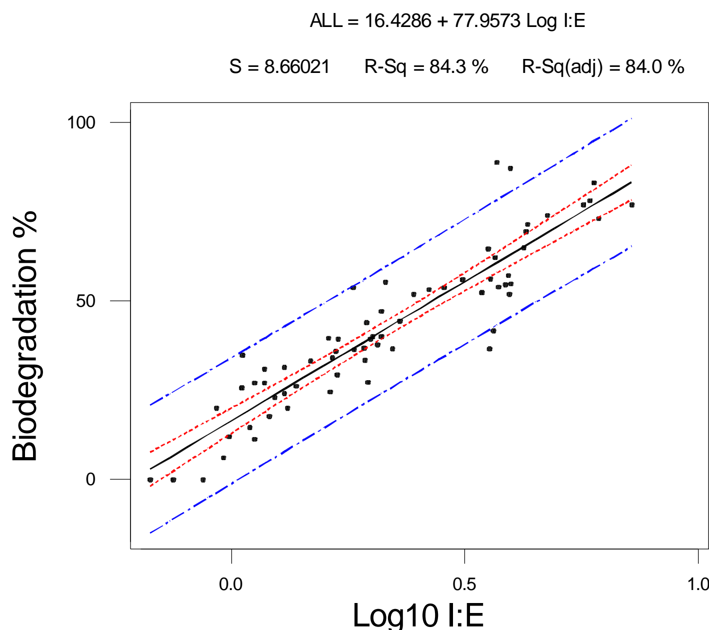

Figure 5. Combined linear regression of % biodegradation of LAB vs. $\log_{10}$ internal:external (*I*:*E*) nC_{12} LAB isomer ratio for all conditions, showing 95% confidence (- - -) and 95% prediction (- — -) intervals

dation. In addition, the use of a logarithmic scale both reduces the effect of this limitation and acts to linearize the resulting plot, making the regression a satisfactory indicator of biodegradation.

The linear fit obtained using the combined data was found to be significant ($R^2 = 0.843$). The regression lines for aerobic and anaerobic biodegradation were checked to see whether they were statistically different by comparing them to this regression (Draper and Smith 1983). The null hypothesis – that the slopes and intercepts of the individual regressions were identical to the composite line – was accepted in every case. The small R^2 value for aerobic flasks ($R^2 = 0.594$, $P < 0.001$) is a reflection of the potential for volatilization of LAB which will tend to overestimate the degradation for a given isomeric ratio. Likewise, the nitrate-reducing flasks show a weak fit ($R^2 = 0.76$, $P = 0.001$) due to small sample size and the restricted degradation due to nitrite accumulation. All the available data were plotted (Figure 5) to yield a regression equation that may be applied to field data where the redox history is unknown.

CONCLUSIONS

LAB is biodegraded in liquid culture under both aerobic and nitrate-reducing conditions. Differential degradation of internal and external isomers follows similar patterns under a range of conditions, while physical and chemical removal does not significantly favour any isomer. The ratio of C_{12} isomers of LAB in the *n*-hexane fraction may provide a robust indicator of the degree of biological degradation at sites that have been contaminated with LAB, even where the redox history of the site is unknown. Biodegradation (*B*, %) can be estimated from the ratio of internal to external isomers of C_{12} LAB by the equation $B = 78 \times \log_{10}(I{:}E) + 16.4$.

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