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Acute Toxicity of Linear Alkylbenzene to *Caenorhabditis elegans* Maupas, 1900 in Soil

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Linear alkybenzene (LAB) is a light non-aqueous phase liquid (LNAPL) whose primary use is as a precursor in the manufacture of linear alkylbenzene sulphonate (LAS) detergents. LAB is also used as insulating oil in underground electricity transmission cables. It has been estimated that some 2×10^6 kg/year of LAB is discharged into the sewage system in Europe (Hansen et al. 2000) and most published data relate to aquatic organisms (Gledhill et al. 1991; Heinze 2001). This work aims to address the lack of data on the toxicity of LAB to terrestrial soil organisms that may be exposed to contamination due to spills of bulk LAB or failure of oil-insulated cables.

MATERIALS AND METHODS

Cable insulating oil was obtained from National Grid Transco. The oil was characterized by GC-MS as consisting of LAB isomers with an alkane chain of between 10 and 13 carbons, and a phenyl group attached to any but the terminal carbon (Johnson 2003; Johnson et al. 2001).

Caenorhabditis elegans (Maupas 1900) is a well-described non-parasitic soil nematode. Its small size, hermaphroditism, short life cycle and the occurrence of a stress-tolerant "dauer" larval stage means that it is easy to raise large numbers of genetically similar, age-synchronised adults in the laboratory (Brenner 1974). The standard method E2172-01 (ASTM 2001) is followed in this work to evaluate the acute toxicity of LAB to *C. elegans* in soil. *C. elegans* wild type strain N2 was cultured in Petri dishes of nutrient agar inoculated with a lawn of *Escherichia coli* OP50 (Lewis and Fleming 1995). Plates were inoculated and incubated at 37ºC overnight prior to use. The dishes of worms were incubated at 20°C. Test animals were taken from a synchronised culture of adult worms prepared as follows: Worms from a 9-day culture were suspended in 3 ml M9 buffer (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 5 g/l NaCl, 0.25 g/l $MgSO_4.7H_2O$) from which 1 ml was removed and pipetted on top of 2 ml of Ficoll suspension (Ficoll 400 diluted to 15% (w/v) in 0.1 M NaCl) in a 15 ml centrifuge tube, taking care not to mix the two layers. The tube was left for 10 min, during which time the active adults migrated to the upper surface, while the relatively immotile dauer larvae sank into the lower layer. The top layer was removed and the dauer larvae washed 3 times with distilled water by centrifuging at 2000 rpm for 2 min and pouring off the supernatant. The dauer larvae were placed on *E. coli* OP50 plates and incubated at 20 °C for 48 h to mature. Adult worms were collected in 5 ml M9 buffer and placed in a 15 ml centrifuge tube. They were washed twice with dH_2O and treated with 7 ml alkaline hypochlorite solution (2 ml Clorox + 5.0 ml 1 M NaOH) for 15 min to kill the gravid adults and lyse the body wall to release the eggs. The tubes were vortexed briefly every 2 min during the alkaline hypochlorite treatment to prevent hypoxia. Eggs were harvested by centrifuging at 1,000 rpm for 2 min and washing the pellet twice in 10 ml M9 buffer. The final pellet was resuspended in 0.5 ml M9 buffer and transferred onto NA plates inoculated with *E. coli* OP50.

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These were incubated at 20°C for 4 d to obtain a synchronised culture of adult worms.

A loam soil (44% sand, 39% silt, 17% clay, total organic carbon 5.8% w/w, pH 5.8) from the Scottish Agricultural College site on the Bush Estate, Edinburgh was used in these tests. It was collected by removing the turf and topsoil to a depth of approximately 20 cm. Subsoil (20-35 cm depth) was dug and kept refrigerated in sealed polythene bags until required for use. Moisture content of the soil was determined by accurately weighing 3-g soil samples before and after drying at 105°C for 24 h or to constant weight. Tests were carried out with a range of concentrations of LAB in soil up to 20,000 mg/kg dry soil (2% w/w). The greatest concentration was mixed first, with lower concentrations being produced by diluting contaminated soil with uncontaminated soil. Soil was first adjusted to 15% moisture content to allow effective mixing with minimum dust. Equipment exposed to the soil was autoclaved. The soil and oil were mixed in a Kilner jar using a custom-made mixer fabricated from 5 mm diameter stainless steel rod. In preliminary tests with this soil, worms were not observed to burrow into the soil at the recommended moisture content of 35% w/w so it was adjusted to 40%. The soils were placed in polythene bags and stored in the dark at 4°C until required. The efficiency of the mixing method was confirmed by taking multiple, 1-g samples and extracting the LAB by shaking in 2 ml hexane. This was quantified by GC-MS using 1-(dodecyl)benzene (a terminal LAB homologue not found in the commercial mixture) as an internal standard (Johnson 2003).

To test the toxicity of LAB to *C. elegans*, three replicates of 2.33 g (dry weight) of each of six concentrations of LAB in hydrated soil, plus uncontaminated controls, were placed in individual 35×10 mm (diameter $\times$ depth) polystyrene Petri dishes. The soil surface was smoothed with a stainless steel spatula to facilitate the introduction of the nematodes. Once prepared, the containers were placed in a sealed container lined with damp absorbent paper. This was stored in the dark at 20 °C for 7 d to allow the soils to equilibrate. Each replicate was populated with 10 nematodes from 3- to 4-d old synchronised cultures. The nematodes were removed under a dissecting microscope by lifting under the body using a flamed and cooled platinum wire. To prevent desiccation of the seed culture, 0.5 ml M9 buffer was pipetted onto the surface of the agar. This was supplemented as required with additional buffer. Individual worms were placed directly on the soil surface and observed to ensure that they burrowed into the soil. Where there was any doubt regarding the health of a worm, it was removed from the soil, the wire was flamed and a new worm was transferred. The test chambers were placed in a closed container lined with damp absorbent paper and incubated for 24 h in the dark at 20°C. The worms were recovered from the soil by differential density flotation in 2 ml colloidal silica (50 ml Ludox HS-40, 50 ml dH_2O, adjusted to pH 7 with conc. HCl). The contents of each test chamber were transferred to a clean 50-ml centrifuge tube. The bulk of the soil was carefully transferred using a clean spatula. Soil adhering to the test chamber was

rinsed with the Ludox suspension. The tubes were capped and vortexed gently in order to suspend the soil, then centrifuged at 2,000 rpm for 2 min and left to settle for 15 to 30 min in order to allow the nematodes to migrate to the liquid surface. The liquid was withdrawn using a 5-ml pipette and placed in a clean Petri dish with a grid ruled on the base to facilitate the search for test organisms. It was inspected under a dissecting microscope and worms were removed using a 200-µl pipette. If the worms were seen to be moving before removal, they were counted as living. If there was any doubt, they were deposited on a lawn of *E. coli* OP50. If after several minutes the worms did not react to gentle stimulation of the anterior end with a platinum wire they were counted as dead, as were any worms not recovered. The mortalities for each of the 3 replicates of each treatment were pooled and the LC50 (*C. elegans*, 24 h) was calculated by Probit analysis (Finney 1964) using the MS-DOS probit.exe program from the USEPA (EPA 2003). The LAB content of the soil remaining in the centrifuge tubes was verified by GC-MS of a hexane extraction with 1-(dodecyl)benzene internal standard. This was done both to verify the relative oil contents of the different concentrations and, by analysis of the isomeric composition, to ensure that no appreciable biodegradation had occurred during the equilibration period (Johnson 2003).

RESULTS AND DISCUSSION

At least 80% of test animals were recovered from all treatments, with a clear increase of mortality with concentration. Mean mortality in the negative control treatments was 30%, which is higher than the 10% recommended in the standard method (ASTM 2001). The oil content and isomeric composition analysis by GC-MS (data not shown) verified that the soil samples were homogeneous and that no measurable biodegradation of LAB occurred during the 24 h exposure period. LAB recovery was 71.3% (standard deviation = 6.99, n = 9). Mortality data (Figure 1) were subjected to Probit analysis (Table 1).

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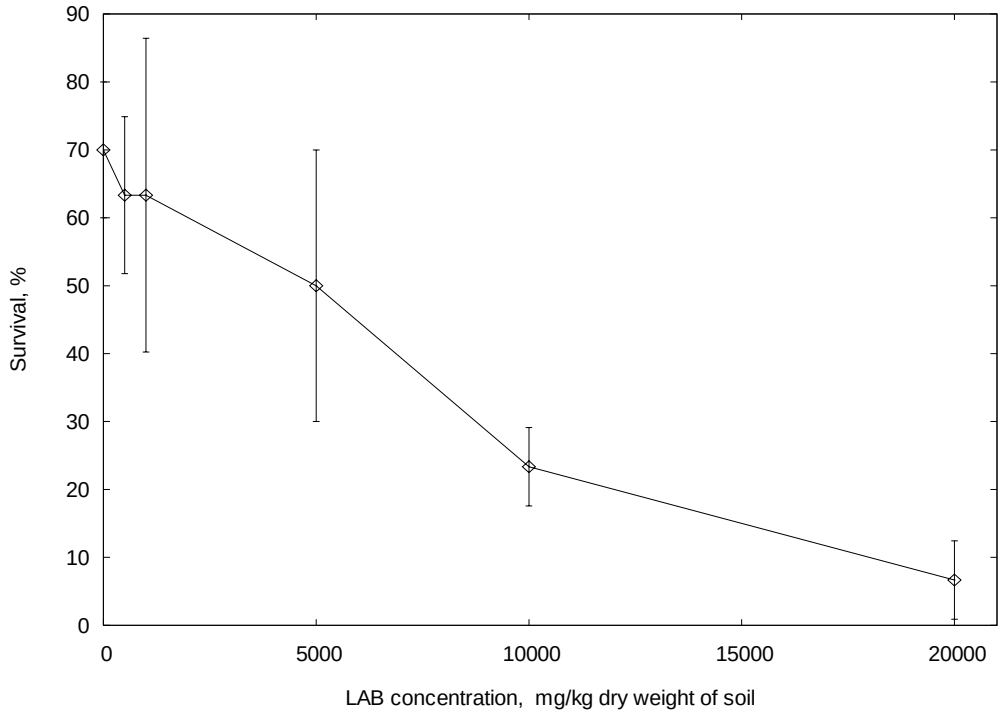

Figure 1 Survival of *C. elegans* exposed to LAB in soil for 24 h. Error bars show 1 standard deviation, n = 3

Table 1 Probit analysis - concentrations of linear alkylbenzene required to achieve a range of lethalities in *C. elegans* after 24 h exposure in a loam soil, with upper and lower 95% confidence limits (CL).

LC	LAB concentration, mg/kg dry weight soil (%)	Lower 95 % CL, mg/kg	Upper 95 % CL, mg/kg
1	1,550 (0.155)	80	3,360
5	2,500 (0.255)	260	4,600
10	3,230 (0.323)	480	5,450
15	3,840 (0.384)	740	6,140
50	7,960 (0.796)	4,060	10,980
85	6,520 (0.652)	11,920	36,800
90	19,630 (1.963)	13,810	54,610
95	25,360 (2.536)	16,770	100,290
99	40,980 (4.098)	23,370	323,790

C. elegans provides an increasingly popular model for toxicology in soil. Its small size and hence large surface area to volume ratio means that it is sensitive to contaminants over a much shorter timescale than, for instance, earthworms. However, this small size does have implications for handling the animals and this was evident in the high mortality seen in control populations. The worms were not fed apart from microorganisms already present in the stored soil. This has been

shown to result in apparently elevated toxicity to metals in liquid culture (Boyd et al. 2003) and so a shorter exposure time may be appropriate. Only a single soil type was used in this investigation, and it is likely that bioavailability and hence toxicity will vary in soils with different clay and organic contents. Soil type has been shown to affect apparent toxicity of metals (Boyd and Williams 2003). Since LAB is often associated with LAS, it is worth noting that surfactants also affect availability of contaminants in soil (Christofi and Ivshina 2002; Providenti et al. 1993; Rouse et al. 1994). Surfactants have been shown to stimulate growth of *C. elegans* (Mutwakil et al. 1997); conversely, their increased availability due to desorption of hydrophobic contaminants may increase toxicity.

The differential density flotation technique used here was developed by Donkin and Dusenbery (1993), who used it to derive concentration-response survival curves and investigate the factors affecting the toxicity of zinc, cadmium, copper and lead in a variety of soils (Donkin and Dusenbery 1994). As an endpoint for a toxicity assay, death is of limited usefulness; it is relatively easy to identify but it does not give any information about the long-term effects of sub-lethal concentrations on the soil community. Multiple endpoints have been identified for nematodes based on lethality, feeding, locomotion and reproduction (Anderson et al. 2001; Anderson et al. 2004; Boyd et al. 2003; Dhawan et al. 1999, 2000). Nevertheless, LC50 remains the most commonly used measure of environmental toxicity. LC50 values determined for *C. elegans* exposed to nitrate salts of cadmium, copper, zinc, lead and nickel in artificial soils for 24 h are similar to those determined for the earth worm *Eisenia fetida* (Peredney and Williams 2000). Other studies also point to *C. elegans* as an alternative to earthworm studies (Boyd et al. 2001), at least for heavy metal toxicity, though some use has been made of *C. elegans* to assess hydrocarbon contamination in soils (Saterbak et al. 1999). More recently, *C. elegans* behaviour has proved to be good model for neurotoxicity studies (Anderson et al. 2004; Cole et al. 2004; Williams et al. 2000). Since this work was performed a variation on the method has been developed that uses a fluorescent transgenic strain of *C. elegans* both to aid recovery of the test organisms and to distinguish them from worms already present in the soil (Graves et al. 2005).

Aquatic and mammalian toxicity data are available for LAB (Gledhill et al. 1991; Heinze 2001). The European Centre for Ecotoxicology and Toxicology of Chemicals suggests that aquatic toxicity data may be of use in estimating soil toxicity (ECETOC 2002) on the basis that the primary source of exposure will be soil pore water and that, generally, bioavailability and thus toxicity of a given substance will be lower in soil than in water due to sorption, aging and other factors; however, there remains a need for real soil data. Limited research has been done into the toxicity of LAB in soil. Unpublished data (*P. Lee, pers. comm.*) indicate that LAB is not lethal to earthworms exposed for 14 d to concentrations up to 1% dry weight soil (10,000 mg/kg) in either sterile or non-sterile soil according to a standard method (OECD 1984). The only formal

guidance on acceptable levels of LAB in soil is given by the Dutch level indicating serious contamination of soil at 1,000 mg kg^{-1} (Pronk 2000), which is below the LC1 level for *C. elegans* found in this study.

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