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Phytotoxicity of branched cyclohexanes found in the volatile fraction of diesel fuel on germination of selected grass species

Gillian MacKinnon *, Henry J. Duncan

Department of Environmental, Agricultural and Analytical Chemistry, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

HIGHLIGHTS

- The first work to highlight the effect of branched cyclohexanes on germination.
- The volatile fraction of diesel fuel causes acute phytotoxic effects on seeds.
- Seed response to diesel fuel contamination varied greatly between grass species.

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ABSTRACT

During a larger study to screen candidate plant species for phytoremediation of diesel fuel-contaminated soils, it was observed that at relatively low levels of diesel fuel contamination, delayed shoot/root emergence and reduced germination was observed for the majority of plant species investigated. It was theorised that these effects were the result of acute phytotoxicity, caused by the volatile fraction of diesel fuel, with results supporting this theory. This finding was investigated further in the current study. Headspace analysis of diesel fuel showed that between 5% and 10% of diesel fuel consisted of compounds that would volatilise at 20 °C, with the most predominant compounds identified being the isomers of xylene (m-, o- and p-), *n*-alkanes (C9–C12) and alkylbenzenes. There were also low levels of toluene, branched cyclohexanes (methyl-, to butylcyclohexane) and alkenes. Of particular interest were branched cyclohexanes as little work has previously been reported on these compounds. To explain the phytotoxic effect of the volatile fraction of diesel fuel and attribute the effect to a specific compound or group of compounds within diesel fuel, seeds were germinated in petri dishes contaminated with a number of pure branched cyclohexanes. An unusual pattern of germination was apparent, with results varying depending on grass species and the length of cyclohexane branching. Results showed ethyl- and butyl-cyclohexane had a significant effect on the germination rate of selected grass species whereas methyl- and propyl-cyclohexane had little effect.

1. Introduction

The use of plants and their associated microorganisms to enhance breakdown of petroleum hydrocarbon pollutants in the soil has gained increasing acceptance internationally as a viable technology to cleanup contaminated soils (Gerhardt et al., 2009; Gaskin and Bentham, 2010). Efforts to remediate petroleum hydrocarbon-contaminated sites, either to mitigate risks of adverse health or environmental effects or to enable site redevelopment are increasing (Vidali, 2001), making this cost effective and environmentally acceptable *in situ* method highly attractive. Rhizoremediation, which is a type of microbially assisted phytoremediation, has emerged as one of the most successful means by which plants can influence the degradation of petroleum hydrocarbon contaminants. A plant can exude as much as 40% of its photosynthate into the soil (Kumar et al., 2006) and it is these sugars, organic acids and larger organic compounds that are used as sources of energy to support 10–100 times more microorganisms per gram of rhizosphere soil than unplanted soil (Lynch, 1990). In addition, the rapid decay of fine plant root material in the rhizosphere provides an important source of microbial nutrition (Leigh et al., 2002) and root growth stimulates oxidative degradation of petroleum hydrocarbons by promoting soil aeration. This complex interaction of roots, root exudates and the microorganisms populating the rhizosphere have been shown to enhance degradation of petroleum hydrocarbon contaminants (Ferro et al., 1998; Binet et al., 2000; Peng et al., 2009; Gaskin and Bentham, 2010; Lu et al., 2010). By selecting appropriate

* Corresponding author. Present address: Department of Chemistry, Scottish Universities Environmental Research Centre, Scottish Enterprise Technology Park, Rankine Avenue, East Kilbride G75 0QF, Scotland, UK. Tel.: +44 (0)1355 270 142; fax: +44 (0)1355 229 898.

E-mail addresses: Gillian.MacKinnon@glasgow.ac.uk (G. MacKinnon), Harry.Duncan@glasgow.ac.uk (H.J. Duncan).

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plant species, increased plant root growth and an extended rhizosphere influence should lead to enhanced breakdown of petroleum hydrocarbons in planted as opposed to unplanted soils.

As part of a larger study on the rhizoremediation of hydrocarbon-contaminated soil, 25 plant species including grasses, legumes, herbs and commercial crops were screened for their ability to germinate in soil contaminated with diesel fuel (Adam and Duncan, 2002). Diesel fuel is a common petroleum hydrocarbon product found contaminating the terrestrial environment. Diesel fuel is classed as a medium petroleum distillate and has a typical carbon range of C_8–C_{26} with the majority of components in the C_{10}–C_{20} range. Diesel fuel is a complex mixture of hydrocarbons with the majority of components consisting of straight chained, branched and cyclic alkanes, as well as aromatic compounds including mono-, di- and poly-aromatic hydrocarbons (PAHs). Of the medium distillate fuels used in terrestrial situations, diesel has the highest content of PAHs and total aromatics (Wang et al., 1990) which make it particularly difficult to remediate. Regardless of this complexity, diesel fuel can be degraded by a number of soil microorganisms making it a likely candidate for bioremediation. The migration of diesel fuel, on entering the terrestrial environment, is limited due to the physical properties of the fuel (Adam et al., 2002). This makes diesel fuel-contaminated soil a prime candidate for rhizoremediation as the contaminant is held in the surface soil and within rooting depth of most plant species.

One prerequisite to rhizoremediation of petroleum hydrocarbon-contaminated soils is that plants are able to germinate and become established in the presence of contaminants. Understanding the influence of petroleum hydrocarbons such as diesel fuel on the early stages of plant development is therefore essential in assessing the potential of a plant species to enhance remediation efforts. The work described in the present study is a continuation of previous research on the influence of diesel fuel on the germinating seed (Adam and Duncan, 2002). This previous research showed that the ability of seeds to germinate in the presence of diesel fuel varied greatly between plant species and even within subspecies. This differential sensitivity to diesel fuel contamination was most clearly illustrated by the family *Gramineae* (grasses). Low germination rates were initially observed in diesel fuel contaminated soil. However, for most of the plant species screened, germination rates subsequently improved, suggesting that whatever was delaying root/shoot emergence was short lived. The hypothesis that acute phytotoxicity, caused by the volatile fraction of diesel fuel, was causing the delayed shoot/root emergence and reduced germination observed in the initial screening experiment was tested in a series of germination experiments. The results supported the hypothesis and further investigations were carried out in the present study.

Plant screening experiments were conducted to determine the germination response of various grass species to the presence of diesel fuel-contaminated soil and where the volatile fraction of diesel fuel surrounding the seed was kept to a minimum. To help explain the phytotoxic effect of the volatile fraction of diesel fuel and attribute the effect to a specific compound or group of compounds, headspace analysis of diesel fuel was carried out to characterise which hydrocarbons would be present. A phytotoxicity bioassay using selected grass species was then used to evaluate the effect of pure petroleum hydrocarbons, identified in the volatile fraction of diesel fuel, on germination and early plant development.

2. Material and methods

2.1. Plant screening experiment

Fourteen species of grass were screened for their ability to germinate in diesel fuel-contaminated soil. Full details of the experimental setup are given by Adam and Duncan (2002). In brief, John Innes seed compost was used as the experimental soil. To obtain an even distribution of diesel fuel in the soil, diesel was mixed with acetone before adding to the soil. This diesel:acetone was then mixed thoroughly through the soil, at appropriate concentrations to provide 25 g and 50 g diesel kg^{-1} contaminated soil and the acetone allowed to evaporate off in a fume cupboard. Uncontaminated controls were prepared by adding acetone only to the soil. Ten grams of uncontaminated, 25 g kg^{-1} and 50 g kg^{-1} diesel contaminated soil were weighed into petri dishes, in duplicate, then seeds of each test grass species were planted in appropriate petri dishes and the soil moistened. The lids were replaced on the petri dishes and the dishes incubated at 20 ± 2 °C in the dark until the majority of seeds had germinated. The developing seedlings were then grown in light conditions at 20 ± 2 °C with a 16 h light/8 h dark cycle. The petri dishes were watered when necessary and the total germination recorded at 7 and 14 d.

2.2. Volatility experiment

Twenty-five Westerwold's ryegrass, Sweet vernal grass and Annual canary grass seeds were planted, in duplicate, in 0, 25 and 50 g diesel kg^{-1} experimental soil as described in Section 2.1. Each petri dish was then set up with an acetate collar supporting the petri dish lid. Holes were put in the lid to allow the volatile diesel components to dissipate whilst allowing moisture to be retained. The petri dishes were incubated under the conditions described earlier and the total germination recorded at 7 and 14 d.

2.3. Headspace analysis of diesel fuel

2.3.1. Headspace sampling procedure

1 g of diesel fuel was weighed into a Chrompack™ headspace vial (glass, 4 cm × 2 cm). The vial was then sealed with a Teflon septum insert and metal collar. The vial was incubated at 20 °C ± 2 °C for 48 h to allow the volatile and non-volatile components to equilibrate. As qualitative analysis of diesel was the objective it was not necessary to determine when the sample reached equilibrium, only when a sufficient gas-phase concentration of the volatile diesel components was reached. A 0.5 ml headspace sample was withdrawn from the vial using a 1 ml gas-tight syringe (JW Chromatography) and injected directly onto the GC column. The sample was analysed using GC–FID and GC–MS as described in Sections 2.3.2 and 2.3.3.

2.3.2. Gas chromatography–flame ionisation detection (GC–FID) analysis of diesel fuel headspace

The method for diesel fuel headspace analysis by capillary GC–FID was based on the US EPA method 8100 for the analysis of polyaromatic hydrocarbons (PAHs) (US EPA, 1986). Analyses were carried out using a Hewlett–Packard 5890A gas chromatograph and Flame Ionisation Detector (FID). Helium carrier gas was adjusted to the recommended linear flow velocity of 20 cm s^{-1}. Separations were performed on a SGE BPX 5 polysilphenylene–siloxane capillary column (25 m × 0.32 mm I.D. × 0.5 μm film thickness). The injection mode was purged splitless. 0.5 ml of diesel headspace was injected onto the column at an initial column temperature of 35 °C with a temperature hold of 3 min. The temperature rose steadily at 5 °C min^{-1} to 250 °C. The temperature was held at 250 °C for 10 min. The injector temperature was 260 °C and the detector temperature 270 °C.

2.3.3. Gas chromatography–mass spectrometry (GC–MS) analysis of diesel fuel headspace

Capillary GC–MS was carried out using a Hewlett–Packard 5971 mass selective detector interfaced to a 5890 series II gas