Phytotoxicity of branched cyclohexanes found in the volatile fraction of diesel fuel on germination of selected 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 5-10% 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 butylcyclohexane had a significant effect on the germination rate of selected grass species whereas methyl- and propylcyclohexane had little effect.

Keywords

Diesel fuel, germination, volatile fraction, phytoremediation, branched cyclohexanes

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 (Gaskin & Bentham, 2010, Gerhardt et al., 2009). 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 (Binet et al., 2000, Ferro et al., 1998, Gaskin & Bentham, 2010, Lu et al., 2010, Peng et al., 2009). By selecting appropriate 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, twenty-five 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 to 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 polyaromatic 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 x 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 hours 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 sec^{-1}. Separations were performed on a SGE BPX 5 polysilphenylene-siloxane capillary column (25 m x 0.32 mm I.D. x 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 minutes. The temperature rose steadily at 5 °C min^{-1} to 250°C. The temperature was held at 250 °C for 10 minutes. 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 chromatograph. Mass spectra were recorded at 70 eV on continuous scanning mode and run through the computer's NBS library for identification. The carrier gas used was helium with a flow rate of 1 ml min^{-1}. Separations were performed on a HP1 fused silica capillary column (12.5 m x 0.2 mm I.D. x 0.33 µm film thickness). 0.5 ml diesel headspace was injected onto the column at an initial column temperature of 35 °C with a temperature hold of 3 minutes. The temperature then rose to 250 °C on a steady 5 °C min^{-1} programme with no temperature hold at the end of the programme. The sample was split at a ratio of 5:1.

2.4 Phytotoxicity Bioassay of Individual Branched Cyclohexanes

0.1, 1 and 5 mg L^{-1} methyl-, ethyl-, propyl- and butylcyclohexane (≥99.0 %, Sigma Aldrich) were individually prepared in acetone (HPLC Grade, Fisher Scientific)**.** 1 ml of each cyclohexane solution at each concentration was added to an appropriately labelled petri dish lined with filter paper (Whatman No.1, 9 cm diameter) which absorbed the added contaminants. The acetone was allowed to evaporate then the filter paper was dampened with water and the seeds placed on the filter paper. Uncontaminated controls were prepared by adding acetone only to the petri dish lined with filter paper. Separate petri dishes containing 15 Westerwold's Ryegrass and 15 Sheep's Fescue seeds were prepared, in triplicate, in the four cyclohexane treatments at three different concentrations. The petri dishes were incubated as described in Section 2.1 and the total germination rate and shoot length measured at appropriate intervals.

2.5 Data Analysis

Time zero was taken to be when the seeds were placed in the petri dishes and germination was considered to have occurred when a sizeable shoot (> 2 mm) and root had emerged from the seed coat. Total germination (% G_T) was determined according to Calvelo Pereira et al., (2010) as:

$$\% G_T = N_g / N_s \times 100$$

where N_g = number of germinated seeds at the end of the experiment and
N_s = number of seeds used in the experiment

2.6 Statistical Analysis

The standard error of the mean was used to provide an estimate of the precision of the sample mean as an estimate of the population mean. Sample standard deviations, calculated using Microsoft Excel statistics were used to work out the standard error of the mean. To ensure the population mean was not underestimated due to the small sample size used during the seed germination experiments (n=2 or n=3), the sample standard deviations were corrected for this bias using the correction factor (approximate values of c_N) of Gurland and Tripathi (1971).

3 Results and Discussion

3.1 Germination of grass seeds in diesel fuel-contaminated soil

Table 1 shows the ability of grass seeds to germinate in diesel fuel-contaminated soil ranged from completely unaffected at lower levels of contamination (e.g. Creeping Bent) to completely susceptible (e.g. Couch grass). Some grass species germinated well (e.g. Westerwold's ryegrass) whilst others were affected initially, resulting in a delayed shoot/root emergence. These species had low percentage germination rates at 7 d but germination increased by 14 d. This effect is illustrated by Sheep's Fescue, with percentage germination being 64 %, 26 % and 6 % at 7 days in 0g, 25 g and 50 g diesel kg^{-1} soil respectively. By day 14, the germination rate had risen to 58 %, 38 % and 24 % in 0g, 25g and 50 g diesel kg^{-1} soil. This trend is also seen with Strong Creeping Red Fescue. The inhibition of germination generally increased with increasing diesel fuel concentration with some species showing an almost linear decrease in germination with increasing diesel fuel concentration (e.g. Black grass). The full set of results for this plant screening experiment including herbs, legumes and commercial crops is presented in Adam and Duncan (2002).

3.2 Phytotoxicity of the volatile fraction of diesel fuel

Selected grass seed species were germinated in petri dishes, in the same levels of diesel fuel-contaminated soil as before (0 g, 25 g and 50 g diesel kg^{-1} soil) except an acetate collar was used to create a volume of air space above the germinating seeds which would allow the diesel fuel volatiles to dissipate. Table 2 shows the results of this experiment. Although the results are variable at 7 d, the total germination rates of the seeds in both treatment levels after 14 d are more similar to the control germination rate. As the volatile fraction of diesel is less concentrated in this experimental set up, the results support the suggestion that the volatile fraction of diesel fuel has an influential role in delaying shoot/root emergence and reducing germination in grasses.

3.3 Analysis and characterisation of the volatile fraction of diesel fuel

As diesel fuel is a complex mixture of both volatile and non-volatile compounds, it was important to determine which compounds would volatilise, under the experimental conditions, from diesel fuel as these compounds were surrounding the seed and influencing germination and early seed

development. A sample of diesel fuel was placed in a sealed vial and the vial stored at 20 ± 2 ^{o}C whereby the compounds, which were volatile at this temperature, began to evaporate from the diesel fuel until they reached equilibrium in the headspace above the sample. Headspace analysis by GC-FID showed that the volatile fraction of diesel fuel at 20 ± 2 ^{o}C made up between 5 and 10 % of the total diesel fuel. This result is consistent with the work of Pichtel & Liskanen, (2001) who found that over 150 d, approximately 10.6 % of diesel range organics was lost by volatilisation.

To identify the volatile hydrocarbons present in the diesel fuel headspace, another diesel sample was prepared for equilibrium headspace sampling and analysed by GC-MS. The chromatographic conditions used for the GC-MS analysis were the same as those used for GC-FID analysis except a different column was used. Very similar patterns of separation were obtained. Of the 35 peaks found by GC-MS analysis, 20 peaks were identified and the other 15 peaks were grouped into their respective hydrocarbon family or sub-family by analysis of the mass spectra (Table 3). The most predominant volatile hydrocarbons found in diesel fuel headspace were the isomers of xylene (m-, o- and p-), short chained n-alkanes (C_9-C_{12}) and other branched benzenes. There were also low levels of toluene, branched cyclohexanes (methyl- to butyl-) and alkenes. The type of toxicity induced by petroleum hydrocarbons is related to the molecular weight of their components, with acute toxicity being induced by low molecular weight components and chronic toxicity induced by high molecular weight components (Gauvrit and Cabanne, 1993). Some work has previously been published on phytotoxicity of volatile hydrocarbons. Low molecular weight n-alkanes (C_6-C_{12}) are non toxic to plants (Crafts and Reiber, 1948) and introducing a double bond to the structure only increases toxicity slightly. These components of the volatile diesel fuel fraction were therefore unlikely to be causing the observed effect on germination. However, phytotoxicity in both vapour and spray treatments is generally exhibited by gasoline and the light ends of oils and increases in the order benzene, toluene and xylene (Currier, 1951) this being consistent with the conclusions of Crafts and Reiber (1948) who found that phytotoxicity increased as the side chain was lengthened in this series. These branched benzene compounds were present in the volatile diesel fuel fraction and were probably also having an effect on germination rate. Such toxicity is violent but non-persistent because the compounds causing the toxicity are relatively volatile and soon leave the plant (Crafts and Reiber, 1948).

However, of particular interest were the components identified in the volatile diesel fuel fraction as branched cyclohexanes as little work has been carried out on these compounds. A homologous series of cyclohexane with increasing branching was identified in diesel fuel by GC-MS analysis (unpublished results) with the shorter branched of these cyclohexanes identified in the headspace. Crafts and Reiber (1948) showed cyclohexane was more toxic that methylcyclohexane when applied as a spray diluted in paraffin oil. However, the activity of hydrocarbons is dependent

on diluent and on a molar concentration basis, a hydrocarbon diluted with air is ~ 30 times more phytotoxic than a hydrocarbon diluted with water and approximately 3,000 times more phytotoxic than if it were diluted in paraffin oil (Currier, 1951). So, although work has been carried out on the toxicity of these hydrocarbons, little is known about their toxicity to germination whilst in the vapour phase. An experiment was therefore designed to test the effect of increasing branching of cyclohexane on the germination of two grass species which showed differential sensitivity to diesel fuel contamination in the initial screening experiments.

3.4 Phytotoxicity of branched cyclohexanes on selected grass species

Two grass species, Westerwold's ryegrass and Sheep's fescue were chosen to investigate the phytotoxicity of branched cyclohexanes present in the volatile fraction of diesel fuel. During the initial screening experiment, both species were able to germinate, albeit at a reduced rate, in the presence of diesel fuel-contaminated soil and their germination response improved with time. Westerwold's ryegrass germinated well whereas Sheep's fescue proved more susceptible to diesel fuel contamination suggesting Sheep's fescue may provide a better indication of the toxic effect of branched cyclohexanes. Grasses such as ryegrass and fescue are commonly used in remediation of petroleum hydrocarbon and PAH contaminated soils (Aprill and Sims, 1990, Binet et al., 2000, Ferro et al., 1998, Cheema et al., 2009) due to their fibrous root system with extensive surface area for microbial colonisation.

In the Westerwold's Ryegrass bioassay, controls started to germinate after 3 d whereas germination was delayed until day 6 in the contaminated treatments. By day 9, measurable shoots and roots had been produced in both control and contaminated treatments (Table 4). The results were varied but showed branched cyclohexanes delayed shoot/root emergence and caused a reduction in shoot length. The observed reduction in shoot length cannot be entirely attributed to the delayed shoot/root emergence, therefore the cyclohexanes must also be having a detrimental effect on growth. Although, no difference was found between the germination results, there was tentative evidence to suggest that increasing levels of methyl-, ethyl- and butylcyclohexane had a detrimental effect on germination but propylcyclohexane seemed to enhance germination slightly at two of the concentrations.

This experiment was repeated using a less tolerant plant species to try and obtain a clearer picture of the effect of branched cyclohexanes on germination. Sheep's fescue seeds were germinated in 0.1–5.0 mg L^{-1} of each branched cyclohexane as before. The results shown in Figure 1 were much easier to interpret. The ethyl- and butyl- branched cyclohexanes had a significant impact on germination rate with few or no seeds germinating in any of the ethylcyclohexane concentrations and a normal germination rate being observed at only the lowest level of butyl cyclohexane. By stark comparison, the methyl- and propylcyclohexanes allowed germination to proceed in the Sheep's fescue seeds. At 0.1 mg methylcyclohexane L^{-1}, there was evidence to suggest that germination appeared to be enhanced by the contaminant and at higher concentration levels the germination rate was just slightly below the control germination rate. Propylcyclohexane reduced the germination rate at 0.1 and 5.0 mg L^{-1} levels but appeared to enhance the germination rate at 1.0 mg L^{-1} level. This unusual result may have been caused by a non-homogeneous selection of seeds being used. When carrying out the germination experiments, a homogeneous selection of seeds was made to provide as even a response to germination and growth as possible. However, it was very difficult to obtain homogeneity for seeds with a husk such as Sheep's fescue.

A similar observation was made in the case of toxicity of substituted benzenes. Crafts and Reiber (1948) found benzene toxicity increased with increasing number of isopropyl substitutions but not in a simple series. The mono- and tri- substitutions were low in toxicity and the di- and tetra-substitutions were high in toxicity. Patterns of substitution can have significant implications for herbicide toxicity, possibly due to differences in their ease of metabolism.

4 Conclusion

This and previous work by the authors have shown that the lower molecular weight, volatile hydrocarbons present in diesel fuel have a large influence on the germination of seeds of some plant species. Although short term phytotoxic effects of petroleum compounds on early seedling growth have been observed by other researchers (Hou et al, 1999, Kroening et al, 2001), to our knowledge, no one has shown that phytotoxic effects can be linked to a specific compound or group of compounds present in the volatile fraction of diesel fuel. Volatile hydrocarbons were shown to delay shoot/root emergence and have a detrimental effect on plant development with branched cyclohexanes shown to be successful at arresting growth in certain grass species. An unusual but consistent pattern of germination was apparent when Sheep's fescue seeds were germinated in the presence of branched cyclohexanes identified in the volatile fraction of diesel fuel, with length of

cyclohexane branching determining toxicity to seeds. The ethyl- and butylcyclohexanes appeared to be extremely phytotoxic to seeds whilst the methyl- and propylcyclohexanes had no or slight phytotoxicity.

The presence of volatile petroleum hydrocarbons in soils at relatively low concentration could severely hinder seed germination resulting in poor plant establishment. This work highlights the importance of fully understanding the influence of the individual compounds or fractions found within petroleum hydrocarbon on the various stages of plant development in order to assess the potential of a plant species to enhance remediation efforts prior to establishment of expensive pot or field trials.

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Tables

Table 1. Total germination (% G_T) of grass species exposed to varying concentrations of diesel fuel, measured 7 and 14 d after planting at 20 ± 2 °C.

Plant species		% G_T*					
		7 d			14 d		
		Diesel concentration g kg $^{-1}$			Diesel concentration g kg $^{-1}$		
Common Name	Latin Name	0	25	50	0	25	50
Cocksfoot	*Dactylis glomerata*	47	17	0	53	20	0
Creeping bent [a]	*Agrostis stolonifera*	20	20	3	30	38	5
Highland bent [a]	*Agrostis castellana*	85	45	45	85	50	46
Common bent [a]	*Agrostis capillaris*	96	32	18	98	34	20
Black grass	*Alopecurus myosuroides*	37	23	3	60	30†	3
Red grass	*Alopecurus pratensis*	20	13	0	17	13	0
Sweet vernal grass [a]	*Anthoxanthum odoratum*	90	55	10	90	60	15
Rough meadow grass [a]	*Poa trivialis*	50	6	0	55	10	0
Westerwold's ryegrass	*Lolium multiflorum*	84	72	46†	78	64	50
Couch grass	*Agropyron repens*	17	0	0	20	0	0
Sheep's fescue	*Festuca ovina*	64	26‡	6	58	38‡	24
Strong creeping red fescue	*Festuca rubra ssp. rubra*	88	68	20	82	88	40
Chewing's fescue	*Festuca rubra ssp. commutata*	56	38†	18	48†	50†	20
Annual canary grass	*Phalaris canariensis*	84	70	14	72	60†	10

* Total germination (% G_T) average values given. Standard deviation multiplied by 1.2500 (approximate value of c_N, n=2, Gurland and Tripathi, 1971), prior to calculation of standard error. Standard error (SE) ≤ 5, n = 2 unless denoted † SE ≤ 10 or ‡ SE ≤ 15.

[a] these seed species were planted at a sow rate of 100 per replicate. The remaining seed species were planted 25 seeds per replicate.

Based on data originally published in Adam, G and Duncan, H (2002).

Table 2. Total germination (% G_T) of grass seed species in diesel fuel-contaminated soil with low volatile diesel fuel components.

		% G_T*					
		Diesel concentration g kg^{-1}					
Plant species		7 d			14 d		
Common Name	Latin Name	0	25	50	0	25	50
Annual canary grass	*Phalaris canariensis*	34	28	54	46	44	60
Sweet vernal grass	*Anthoxanthum odoratum*	34	26	22	38	34	28
Westerwold's ryegrass	*Lolium multiflorum*	78	72	62	80	80	80

* Total germination (%G_T) average values given. Standard deviation multiplied by 1.2500 (approximate value of c_N, n=2, Gurland and Tripathi, 1971), prior to calculation of standard error. Standard error (SE) < 5, n = 2

Table 3. Volatile hydrocarbons identified in diesel headspace by GC-MS.

Retention Time (min)	Peak Area (%)	Compound*
1.417	3.217	**methyl cyclohexane**
1.788	2.041	**toluene**
3.154	1.650	**ethyl cyclohexane**
3.672	3.417	**o-xylene**
3.903	9.120	**m-xylene**
4.270	3.981	branched alkane
4.470	5.968	**p-xylene**
4.702	3.937	iso-cyclohexane
5.313	7.681	**n-nonane**
5.474	1.487	alkyl benzene
5.921	2.466	**propyl cyclohexane**
6.112	1.192	**nonene**
6.364	3.154	alkyl benzene
6.623	4.040	alkyl benzene
6.852	1.036	alkyl benzene
7.359	1.506	**decene**
7.577	3.005	**ethyl benzene**
7.728	1.102	**(ethylpropyl) cyclopentane**
8.157	0.426	alkyl benzene
8.372	0.620	**(methylethyl) benzene**
8.484	3.895	**decane**
8.643	0.373	alkyl benzene
9.151	1.107	**butyl cyclohexane**
9.245	0.861	**ethyl heptane**
9.468	0.600	alkyl benzene
9.566	1.603	alkyl benzene
9.883	0.349	alkyl benzene
10.422	0.681	alkyl benzene
10.543	0.550	**methyl octane**
10.720	0.391	branched alkane
10.861	0.305	branched cyclohexane
11.640	1.913	**n-undecane**
12.178	0.531	branched alkane
12.378	0.381	**(methylpropyl) cyclohexane**
14.639	0.464	**n-dodecane**

Peak area identified = 75 % of total peak area with branched cyclohexane (methyl-, ethyl-, propyl- and butyl-) equalling 11 %, isomers of xylene (m-, o- and p-) equalling 25 % and n-alkanes (C9-C11) equalling 18% of total peak area identified.

* compounds in bold were positively identified whereas remaining compounds were grouped into their respective hydrocarbon family or sub-family.

Table 4. Total Germination (% G_T) and shoot lengths for Westerwold's ryegrass grown in varying concentrations of branched cyclohexanes for 9 d.

Cyclohexane Treatment	Concentration (mg L^{-1})	% G_T*	Shoot length (cm)
control	0	64	63.5 ± 2.27
methyl	0.1	62†	8.50 ±1.02
	1.0	64	8.53 ± 0.82
	5.0	58	7.95 ± 0.25
ethyl	0.1	53	8.73 ± 1.25
	1.0	62	8.80 ± 0.77
	5.0	44‡	7.50 ± 0.58
propyl	0.1	73†	9.53 ± 1.37
	1.0	60	9.10 ± 1.34
	5.0	69	9.73 ± 0.21
butyl	0.1	47	9.20 ± 0.69
	1.0	49	9.60 ± 0.58
	5.0	62†	8.17 ± 0.83

* Total germination (% G_T) average values given. Standard deviation multiplied by 1.1250 (approximate value of c_N, n=3, Gurland and Tripathi, 1971), prior to calculation of standard error. Standard error (SE) ≤ 5, n = 3 unless denoted † SE < 10 or ‡ SE <15.

Average shoot length values given ± SE, n = 3.

Figure captions

Figure 1. Total Germination (% G_T) for Sheep's fescue grown in varying concentrations of branched cyclohexanes for 17 d.

Total germination (% G_T) average values given ± Standard Error, n = 3. Standard deviation multiplied by 1.1250 (approximate value of c_N, n=3, Gurland and Tripathi, 1971), prior to calculation of standard error.

Figures

Figure 1