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Metabolic responses of microbiota to diesel fuel addition in vegetated soil

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Abstract

The effects of trees and contamination on microbial metabolic activity, especially that of hydrocarbon degrading bacteria, were compared during phytoremediation to find which conditions increase diesel fuel removal. Diesel fuel utilisation, microbial extracellular enzyme activities and utilisation of Biolog ECO plate carbon sources by soil bacteria were determined during phytoremediation experiments consisting of two separate diesel applications. Diesel fuel removal after 28 days of second diesel application was 20–30% more than after the first application 1 year earlier. Soil microbiota utilised 26–31 of the 31 Biolog ECO plate carbon sources. Carbon source utilisation profiles indicated minor differences in microbiota in soil vegetated with pine compared to microbiota in soil vegetated with poplar. The potential maximum rates of aminopeptidase activity were 10–10^2 μM AMC/h/g dry soil prior to and after second diesel application, except 14 days after the second diesel addition, where the rates were at the scale of 10^3 μM AMC/h/g dry soil. The potential maximum rates of esterase activity were 10^3–10^4 μM MUF/h/g dry soil. The presence of plants did not influence the activity of esterases. The utilisation of diesel by soil bacteria in Biolog MT2 plate assay was higher in contaminated soil, especially when vegetated, than in uncontaminated soil, measured both as lag times and maximum specific utilisation rates. MT2 plate assay detected the biological response after diesel fuel addition better than general activity methods.

Abbreviations: AMC – 7-amino-4-methylcoumarin; MEE – microbial extracellular enzyme; MUF – methylumbelliferone; PCA – principal component analysis

Introduction

Phytoremediation uses plants to remove, contain or render harmless environmental contaminants. Plants or plant-associated microflora convert many pollutants to non-toxic forms (Cunningham & Berti 1993; Cunningham et al. 1995). In addition, the organic and inorganic substances exuded from the plant roots (Anderson et al. 1993) may favour microbial activity and biodegradation in the root zone. Thus, phytoremediation can be used to enhance bioremediation of contaminants, such as hydrocarbons.

The bioremediation potential of soil can be investigated by following the biodegradation of the pollutants or radio labelled compounds, screening genes coding for pollutant degradation and microbial enumeration of contaminant degraders. Microbial diversity in soil can be investigated with carbon source utilisation patterns. Carbon source utilisation patterns of hydrocarbon-contaminated soil differ from those of uncontaminated soils (Dobler et al. 2000; Wünsche et al. 1995). Furthermore, metabolic profiles of rhizosphere microbial communities differ from bulk soil (Baudoin et al. 2001).

Plants and microorganisms release extracellular enzymes to soil. Land management (Kandeler et al. 1999; Naseby & Lynch 2002) and vegetation (Broughton & Gross 2000) influence soil enzymatic activities. Reports about enzyme kinetic parameters, such as V_{max} and K_m, for soil extracellular enzyme activities are, however, limited. In most studies, only the enzyme activity at one fixed concentration is reported. In the present study the kinetic approach was applied to determine the applicability of Michaelis–Menten kinetics and to determine V_{max} and K_m.

Diesel fuel utilisation, microbial extracellular enzyme activities and carbon source utilisation patterns of soil microbiota were determined during phytoremediation experiment in soil contaminated with diesel fuel. The purpose of this study was to use these methods as a tool to compare the effects of trees and contamination on microbial metabolic and biocatalytic activity, especially that of hydrocarbon degrading bacteria and cleaving of ester, glycosidic and peptide bonds.

Materials and methods

Phytoremediation experiment

Soil material used in a growth chamber study was collected from A and B horizons of a mixed coniferous forest in Tampere, Finland. The soil was mixed and sieved through a 3-mm mesh sieve. The soil was classified as sand and the chemical and physical properties of the soil were as presented by Palmroth et al. (2002). Each pot was planted with a 1-year old seedling of Scots pine (*Pinus sylvestris*) or Poplar (*Populus deltoids x Wettsteinii*). In addition to plants, four different soil amendments were utilised, including a commercial mixed nitrogen–phosphorus–potassium fertiliser, compost extract, microbial enrichment culture, and no amendments (Table 1). The compost extract was prepared from matured biowaste compost. Four hundred gram of sieved compost and 1 l of distilled water were mixed in a water bath at 40 °C for 5 h and the decanted liquid was applied. Total organic carbon of the filtered (0.45 μm) extract was 700 mg/l. For the microbial enrichment culture treatment, a total of 1.6×10^{10} cells (DAPI total cell count) was applied per pot. The microbial enrichment medium was prepared from DSM 465 mineral medium (DSMZ, German Collection of Microorganisms and Cell Cultures), in which magnesium chloride was replaced with magnesium sulphate. 0.6% of diesel fuel was used as sole carbon source and 0.02% of the same Tampere soil material was used as inoculum.

Fresh diesel fuel (Neste Futura) was mixed with the soil throughout the pot at an initial concentration of 0.5% (w/w) as presented in Palmroth et al. (2002). Four replicates of each plant treatment/soil amendment were used. The effect of supplementation with 2% diesel fuel was studied in a 1-month experiment 1 year after the first diesel fuel addition. In this event, the diesel fuel was applied to the soil surface. The trees and soil were stored outside over the winter season prior to the second application. Control pots without vegetation were used to determine the effect of the plant on diesel fuel degradation and microbial activity.

All of the soil was removed from the pots and separated from roots by manual shaking and by use of a surgical blade. The soil was mixed thoroughly with a spoon and subsamples were taken for analyses.

The diesel fuel extraction procedure was as presented by Palmroth et al. (2002). Briefly, soil was extracted with hexane in an ultrasonic bath and extracts were analysed with gas chromatograph with mass selective detector. The soil, stored at 6 °C, was extracted within 1 day of collection. Soil moisture content was determined by measuring weight loss after oven-drying at 105 °C and the diesel fuel concentrations in soil are reported on a dry weight basis.

Soil suspension

The mixed soil samples (2 g subsample) were first diluted 1/100 with sterile deionised water (Milli-Q®) and shaken on a rotary shaker at 150 rpm overnight. Soil suspensions were prepared in deionised water, because it does not contain nutrients interfering with extracellular enzyme analysis and because buffering may change the enzymatic activities as well as the bioavailability of contaminants and substrates. Soil suspensions were shaken manually prior to usage in order to keep particulate matter dispersed in the liquid phase.

Table 1. Soil amendments added to 500 g of soil

Amendments	Addition	Details
NPK fertiliser	25 mg	16.6% N, 4% P and 25.3% K
Compost extract	5 ml	Biowaste compost extract, TOC 700 mg/l
Microbial enrichment culture	1.6×10^{10} cells (DAPI total cell count)	0.6% of diesel fuel and 0.02% sieved soil added in DSM 465 mineral medium, incubated 2 weeks on a rotary shaker (150 rpm) at 20–22 °C

Diesel and Biolog ECO plate carbon source utilisation of soil microbiota

Carbon source utilisation of soil microbial communities were assessed using Biolog ECO (Biolog 2000) and MT2 plates (Biolog Inc., Hayward). ECO plates contain 30 carbon sources with three replicas, redox dye and buffered nutrient medium, while MT2 plates contain only the redox dye and buffered nutrient medium. A 90 μl aliquot of soil suspension was injected into each of the 96 wells of the plates.

In this study, the utilisation of the volatile fraction of diesel fuel was used as an indicator of diesel fuel degradation. Diesel fuel was allowed to evaporate into a desiccator atmosphere and the Biolog MT2 plates without lid were incubated in the closed desiccator, thus exposing the plates to the maximum vapour pressure of diesel fuel volatile compounds. MT2 control plates filled with soil suspension and sterile water controls were incubated in a desiccator without carbon sources. MT2 plates and controls were measured at least for 2 weeks or until the absorbance reached 2.0 in some of the wells. Absorbance was measured at 590 nm with a Victor™ Multilabel reader (Wallac, Turku, Finland).

ECO plates were incubated with closed lids at room temperature (20–22 °C) in the dark and the sterility of the ECO plate batch was tested by inoculating several plates with autoclaved deionised water. Absorbance at 590 nm in ECO plate wells was measured until day 6 with a Victor™ Multilabel reader (Wallac, Turku, Finland).

Each ECO plate carbon source was scored positive if the standard deviation of absorbance was smaller than the average absorbance. The absorbances were statistically analysed (MATLAB Release 12) with principal component analysis (PCA) and analysis of variance. The first two vectors of PCA are plotted. The vectors obtained in analysis are perpendicular to each other. Thus the first to vectors can be plotted as axis PCA1 and PCA2. The percentage of total variance explained is reported.

Inoculum densities of Biolog plates were not standardised with the average well colour development method (Garland & Mills 1991), because this standardisation method decreases inter-sample distances in PCA (Howard 1997). Statistical analyses of absorbances were carried out at 2 and 3 day incubations. Natural logarithms of MT2 plate absorbance divided by MT2 plate absorbance at time zero in a time series were used to calculate the lag time (λ), asymptote (A) and maximum specific diesel utilisation rate (μ_m). A modified Gompertz equation $y = A\exp\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\}$ (Zwietering et al. 1990) and nonlinear least squares data fitting in MATLAB Release 12 by the Gaussian–Newton algorithm with Levenberg–Marquardt modifications for global convergence were used in the data analysis.

Viable plate counts of soil bacteria and actinomycetes

Viable plate counts in soil were determined prior to the transfer of pots outside for the winter and after the 1-month experiment by the spread plate method from soil dilutions to soil extract agar (Burlage et al. 1998) and starch casein medium (Wellington & Toth 1994). The soil dilutions were serially diluted from the soil suspension. The plates of each dilution were prepared in duplicate. Colonies were counted after 7 days of incubation at 20 °C.

Extracellular enzymatic activity of soil microbiota

The effect of plant and diesel fuel contamination on the activity of hydrolytic enzymes in the soil

samples was determined using fluorogenic substrates. The stock and working solutions of enzyme substrates and the model fluorogenic molecules, 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC), were prepared in dimethyl sulfoxide, due to the low water solubility of the compounds. The activity of fourteen enzymes taking part in the hydrolysis of C, N, and P compounds was determined in a 96-well assay. The enzyme substrates and MUF and AMC were purchased from Sigma-Aldrich. The concentrations of model substrates in the assay were 0.5–200 μM for 4-MUF-phosphate, 4-MUF-β-xyloside, 4-MUF-β-D-*N*-acetyl-glucosamide and 4-MUF-cellobiose, 1–500 μM for 4-MUF-α-glucoside, 4-MUF-β-glucoside, 4-MUF-β-galactoside, 4-MUF-butyrate, 4-MUF-palmitate, 4-MUF-stearate, L-leucine-7-AMC, L-alanine-7-AMC and L-Serine-AMC hydrochloride and 2–1000 μM for 4-MUF-acetate. Standards of MUF and AMC (0.001–50 μM) were prepared in duplicate in soil suspensions i.e. standard curves were prepared separately for each sample to take the possible quenching of fluorescence into account. The soil samples were first suspended 1:50 in sterile deionised water (Milli-Q®) and sonicated (5×1 min). The soil suspension (90 μl) was transferred to each of the wells in a 96-well microtiter plate. Our assay was similarly designed as that of Vepsäläinen et al. (2001), except that we used unbuffered soil suspension to prevent changes in nutrient ratio (C, N, P) conditions that may affect the investigation of the potential enzyme activity at field conditions and they used only one concentration of each substrate.

The fluorescence of the hydrolysed model substrates and standards was measured at excitation 355 nm/emission 460 nm with a fluorometer (Fluoroskan Ascent FL, Thermo Labsystems), immediately after addition of soil suspension and at least once during incubation. The rates of hydrolysed enzyme substrate [μM MUF/h/g] or [μM AMC/h/g] at 3 h incubation time were calculated for dry soil to enable comparison of soil samples with different moisture content. Enzyme kinetics was applied to achieve substrate saturation levels to measure the potential maximum rate of microbial extracellular enzyme hydrolysis referred in text as V^*_{max} and apparent Michaelis–Menten parameter referred in text as K^*_m. The term potential is used in this context due to the potential interference of the soil matrix to our applied assay. The V^*_{max} and K^*_m values were calculated from Michaelis–Menten plots with SigmaPlot™ Enzyme Kinetics Package 1.1.

Results and discussion

Diesel fuel removal

Diesel fuel removal was greater in poplar treatment, up to 86% by day 28, than pine treatment after the second diesel fuel application (Figure 1). The diesel fuel removal was faster than after the first diesel fuel application, where only 50–60% of diesel fuel had been removed in 30 days (Palmroth et al. 2002). The higher diesel fuel removal in poplar-vegetated soil maybe related to the fine root structure of poplar compared to the tap root of pine, but to our knowledge studies with pine and diesel contaminated soil are limited to our earlier study (Palmroth et al. 2002).

Part of the observed diesel fuel removal, especially that of short-chain alkanes, is attributed to volatilisation. The applied extraction method is suitable for recent contamination, but the recovery of diesel fuel may be reduced during aging process, where the contaminants are sorbed to soil matrix. During this short 1-month experiment, diesel fuel removal unvegetated soil was equal to diesel fuel removal in pine-vegetated soil, but according to our 1-year experiment (Palmroth et al. 2002) the removal in unvegetated soil is slower in longer time scale.

Diesel fuel utilisation

An assay to measure diesel fuel utilisation with Biolog MT2 plates was developed. The purpose was to create assay describing bioremediation potential which is easier to perform than enumeration of diesel fuel degrading microorganisms by most-propable-number methods demanding preparation of multiple dilutions. Insolubility and toxicity of compounds can limit applying MT plates for substrate utilisation tests (Fulthorpe & Allen 1994).

Diesel fuel utilisation was studied 1 year after the first and the second diesel additions to determine if vegetation or contamination enhances diesel utilisation. Table 2 shows the maximum

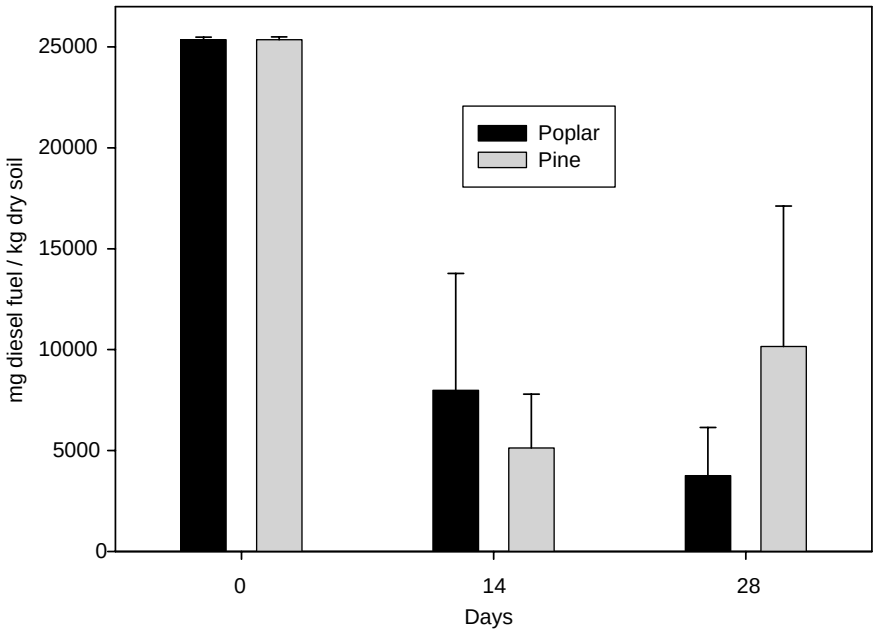

Figure 1. Diesel fuel removal in poplar and pine treatments after the second diesel fuel application (2% w/w) 1 year after the first diesel fuel application (0.5% w/w). Error bars indicate standard deviation of 8 replicate soil samples.

Table 2. Gompertz parameters: maximum specific diesel utilisation rate μM (1/h) and lag time λ(h) of diesel fuel utilisation with confidence limits

Days from 2nd diesel fuel addition	Parameter	Poplar vegetated soil	Pine vegetated soil	Unvegetated soil
Before	λ(h)	96 ± 53	171 ± 126	ND
	μ_m(1/h)	$7.1 \times 10^{-3} \pm 4.1 \times 10^{-3}$	$6.2 \times 10^{-3} \pm 3.2 \times 10^{-3}$	ND
14 d	λ(h)	66 ± 15	66 ± 24	90 ± 207
	μ_m(1/h)	$1.3 \times 10^{-2} \pm 6.6 \times 10^{-3}$	$7.1 \times 10^{-3} \pm 1.5 \times 10^{-3}$	$5.3 \times 10^{-3} \pm 5.5 \times 10^{-3}$
28 d	λ(h)	77 ± 37	85 ± 37	156 ± 7
	μ_m(1/h)	$4.8 \times 10^{-3} \pm 1.5 \times 10^{-3}$	$3.8 \times 10^{-3} \pm 0.7 \times 10^{-3}$	$9.9 \times 10^{-3} \pm 7.2 \times 10^{-3}$

Twenty four or more wells were inoculated with each sample. Number of replicate tree soil samples = 4. Probability (P) that natural logarithmic absorbance ln(absorbance at time t/absorbance at time 0) is similar at all the incubation times was less than 10^{-7}. ND = not determined because data did not fit to Gompertz equation.

specific diesel utilisation rates (h^{-1}) and lag times (h) of diesel fuel contaminated soil. In the uncontaminated soil, both vegetated and unvegetated, the diesel utilisation rates were lower than in contaminated soil and the data could not be fitted to the Gompertz equation due to minor changes in the absorbance. The diesel utilisation rates were higher 14 days after the diesel addition than at other sampling times.

The lag times were longer prior to than after the second diesel application. Thus, diesel fuel utilisation was inducible, and the lag time increased as the time from diesel application elapsed. Presence of plants alone does not increase diesel fuel utilisation in clean soil, but in contaminated soil the presence of trees enhances diesel fuel utilisation. The assay indicated that growth on diesel fuel increases more in the presence of poplar than pine, which is in accordance with diesel fuel removal (Figure 1).

Diesel fuel utilisation assay describes the ability of soil microbiota to utilise the most volatile

fraction of diesel fuel. The abundance of the bacteria capable of degrading the volatile fraction is dependant on the concentration of these compounds in soil. Thus the utilisation is dependant on the abundance of volatile diesel degrading bacteria. The most volatile fraction of diesel fuel, ranging from nonane to dodecane, was removed within 30 days (Palmroth et al. 2002). To our knowledge, the incubation of MT2 plates with volatile contaminant has not been applied to soil, but the procedure has been used to screen the potential of microbial isolates to degrade individual volatile compounds (Strong-Gunderson & Palumbo 1994).

Carbon source utilisation patterns

Carbon source utilisation patterns of soil microbiota were assessed during phytoremediation experiments to determine if contamination and plant alter the microbial diversity in soil. We wanted to explore whether root exudation and addition of diesel fuel change microbial community structure, thus resulting in differences in substrate utilisation.

MT2 plates without carbon sources were inoculated with soil suspension and incubated to determine if the natural organic matter of the soil suspension interferes with the absorbance measurements. The well readings were not significantly different ($p < 0.05$) from the readings prior to incubation; thus, the soil suspension did not contain sufficient amounts of carbon sources to adversely affect the experimental procedure.

The number of utilised ECO plate carbon sources was calculated for each soil sample to find out if the carbon sources in the form of root exudates and diesel fuel change the microbial community structure. The microbial communities of soil samples utilised 26–31 of the 31 carbon sources in Biolog ECO plates. Soil contamination did not reduce number of utilised substrates. Thus, ECO plate substrate utilisation diversity did not change as a result of diesel addition.

In PCA plot of the carbon source utilisation patterns of soil samples taken before the second diesel application, pine- and poplar-vegetated soil samples were clustered separately from the soil without vegetation (Figure 2), but differences between soil treatments were minor. Addition of diesel fuel changed the microbial communities in the soil: soil with no vegetation could be differentiated from samples vegetated with trees, but pine and poplar samples were clustered closer together (Figure 3). However, the change may not be permanent since the effects of the first diesel fuel application were not detected 1 year later. Separation of the rhizosphere compartments (Baudoin et al. 2001) or adding compounds existing in soils, such as root exudates, to MT2 plates (Campbell et al. 1997), could improve the ability of carbon source utilisation patterns to detect changes in soil microbial communities.

Microbial extracellular enzymatic activity

Microbial extracellular enzyme activities were assessed during phytoremediation experiment in soils contaminated with diesel fuel compounds and in control soils to determine how plants affect microbial activity.

Background fluorescence caused by particulate matter in soil suspension changed slightly during incubation. However, this is in contrast with the findings of Marx et al. (2001), according to whom the background absorbance of buffered soil suspension does not change during incubation. Therefore, they calculated concentrations using only the initial readings. Settling of the particulate matter to soil may explain the change and, in the present study, subtracting background fluorescence and making a separate standard curve at each incubation time allowed determination of released MUF or AMC. This calculation also allows to taking possible fluorochrome sorption to soil into consideration, since MUF and AMC had their own standard curves in soil samples at each incubation time. All the standard curves produces were linear and in the same scale as MUF or AMC without soil suspension. However, some sorption may have occurred, since the standard curves changed slightly during incubation, but the difference in the standard curves was not statistically significant.

Apart from results obtained with short-chain organic acid and amino acid substrates, the cleavage of the fluorogenic substrates, 4-MUF-phosphate, 4-MUF-β-xyloside, 4-MUF-β-D-*N*-acetyl-glucosamide, 4-MUF-cellobiose, 4-MUF-α-glucoside, 4-MUF-β-glucoside, 4-MUF-β-galactoside, 4-MUF-palmitate and 4-MUF-stearate was so low that kinetic parameters could not be determined, indicating that the activity of the corresponding

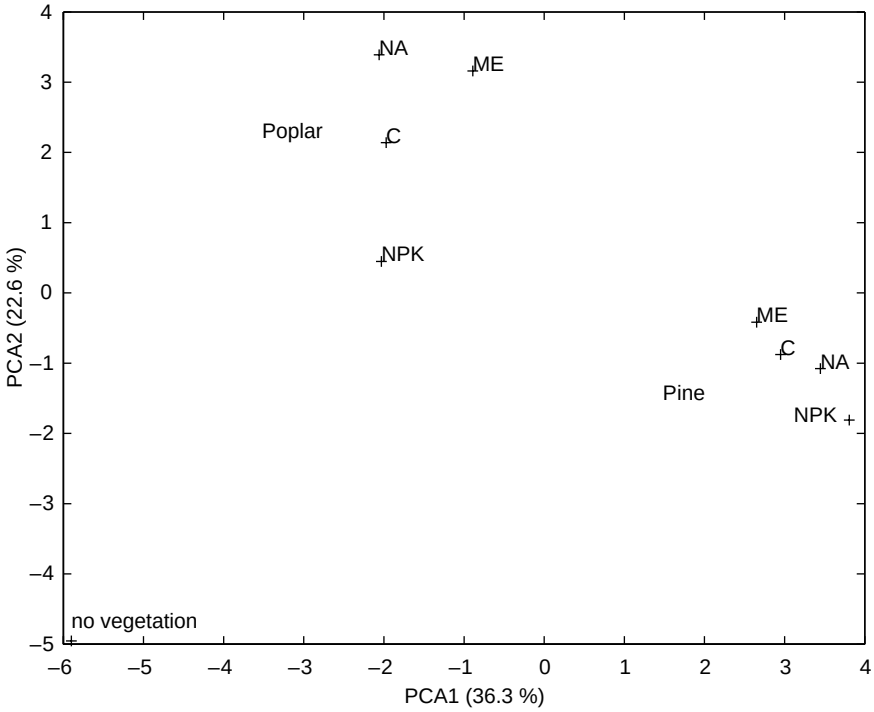

Figure 2. Principal component analysis of measurements obtained from ECO plates incubated for 3 days. Vegetated soil samples, pine or poplar, were contaminated with diesel fuel 1 year prior to assay. Variance explained by each PCA axis presented in brackets. Abbreviations used C compost extract, ME microbial enrichment, NPK NPK fertiliser and NA no additions.

hydrolytic enzymes is low. The low activity of hydrolytic glycolytic enzymes may be related to abundance of sufficient labile organic carbon in soil or inhibition effects. Low phosphomonoesterase activity could indicate that the soil bacteria were not undergoing P limitation. Higher activities were obtained from esterase and aminopeptidase which have been shown to correlate with living biomass measured as adenosine triphosphate (Kähkönen 2003). Therefore, we used these two group of enzyme classes in our study to assess general biocatalytic activity of soil microbiota.

One year after the first diesel spill, the enzymatic activities of serine and alanine aminopeptidases, indicated by V^*_{max}, were lowest in soil vegetated with poplar and treated with nitrogen-phosphorus-potassium fertiliser (Figure 4). The V^*_{max} of leucine aminopeptidase followed the same pattern as the V^*_{max} of serine aminopeptidase (Figures 4 and 5). The highest measured V^*_{max} was in the poplar-vegetated soil with microbial enrichment culture. The V^*_{max} of serine, leucine and alanine aminopeptidases was much higher, 10^3 μM AMC/g dry soil/h, 14 days after the second diesel fuel addition than at other sampling times (Figure 5). Twenty eight days after the second diesel application, the rates in uncontaminated vegetated soil were lower than in contaminated vegetated soil. In general, higher rates were obtained with 7-AMC-alanine than with two other AMC-substrates.

The activities of acetate and butyrate esterase did not differ significantly ($p < 0.05$) from each other and may reflect similar substrate pools. Thus only the V^*_{max} of acetate esterase is presented in Figures 4 and 5. One year after the first diesel spill, the V^*_{max} of acetate and butyrate esterases was higher in fertilised poplar-vegetated soil than in unfertilised poplar-vegetated soil. The potential maximum rate V^*_{max} of both acetate esterase and butyrate esterase was higher 14 days after the second diesel fuel addition than at 28 days after the addition (Figure 5). In diesel fuel contaminated unvegetated soil V^*_{max} never exceeded 10^3 μM MUF/h/g dry soil. Organic acids in soil in form of ester bonds to soil organic matter could partly explain the esterase activities. Usually the

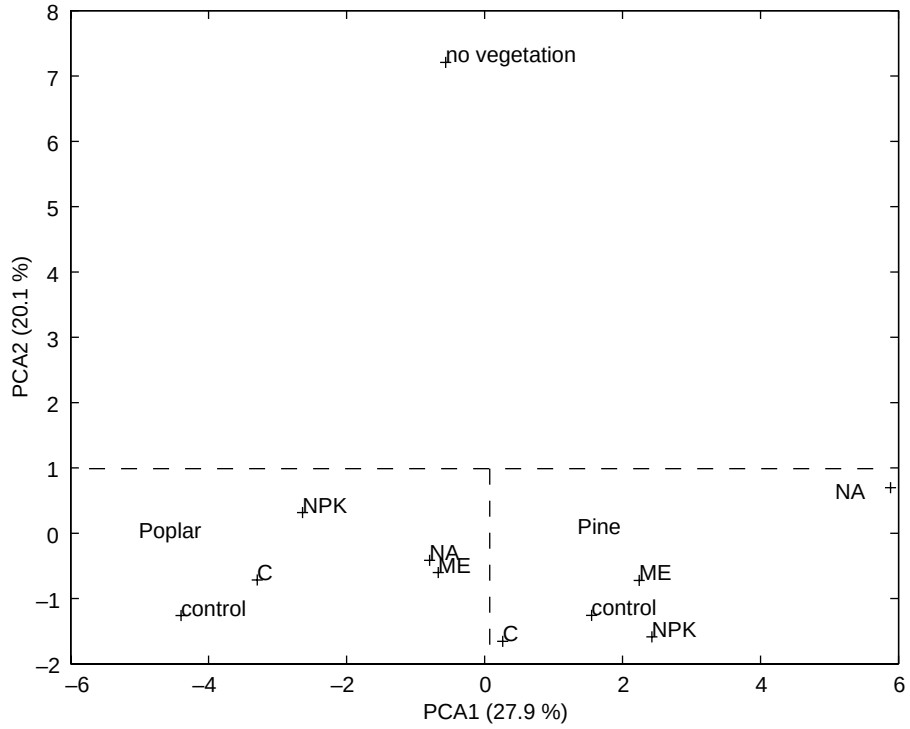

Figure 3. Principal component analysis of measurements obtained from ECO plates incubated for 3 days. Soil samples collected 2 weeks after the 2nd oilspill. Variance explained by each PCA axis presented in brackets. Abbreviations used C compost extract, ME microbial enrichment, NPK NPK fertiliser and NA no additions.

concentrations of the dominant free organic acids, e.g. acetate, oxalate and citrate, in soil solution are <1–100 μM (van Hees et al. 2002) and the abundance of low molecular weight organic acids is higher in rhizosphere soil than in bulk soil (review by Strobel 2001).

Michaelis–Menten K_m^* values of esterases and aminopeptidases were as follows: acetate esterase from 10 to 370 μM MUF, butyrate esterase from 10 to 880 μM MUF, serine aminopeptidase from 8 to 270 μM AMC, leucine aminopeptidase from 3 to 270 μM AMC and alanine aminopeptidase from 8 to 440 μM. The values did not show any trend based on contamination or presence of vegetation, possibly due to the unspecific binding of the enzyme substrates in soil. Further, sorption of extracellular enzymes to minerals or humic substances alters reaction efficiency (Quiquampoix et al. 2002; Tate 2002).

Diesel fuel application did not decrease the extracellular enzymatic activities of esterases and aminopeptidases. Enzymatic activities are more sensitive to metal contamination than to hydrocarbon contamination (Brohon et al. 2001), possibly due to only minor interactions of hydrophobic hydrocarbons with proteins in soil solution (Speir & Ross 2002).

Viable plate counts in soil

Viable plate counts on soil extract agar and starch casein medium were followed to determine if culturable organisms are more abundant in vegetated or recently contaminated soil than in uncontaminated soil. One hundred twenty days after the first diesel application to soil, viable plate counts of bacteria on soil extract agar were at the same level, 3.1×10^6 to 1.4×10^7 cfu/g dry soil and viable counts of culturable actinomycetes on starch casein medium were at the same level, 3.8×10^6 to 3.1×10^7 cfu/g dry soil, regardless of vegetation type or presence of contamination. After the second diesel application, viable plate counts on soil extract agar ranged from 3.1×10^6 to 3.1×10^7 cfu/g dry soil. Culturable actinomycetes (starch casein medium) had viable counts of

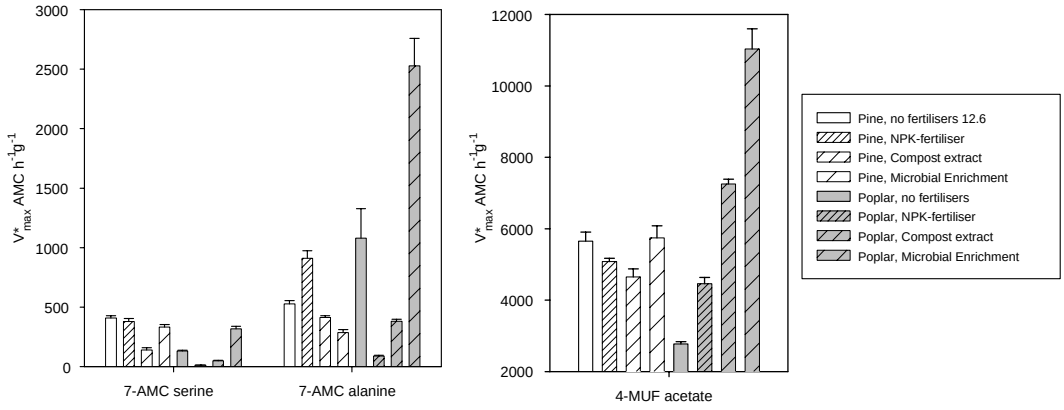

Figure 4. Potential maximum rate of enzymatic activity V^*_{max} of serine and alanine aminopeptidases (μM AMC/h/g dry soil) and acetate esterase (μM MUF/h/g dry soil). Activities measured in pine and poplar vegetated soil with different soil amendments 1 year after the first diesel fuel addition. Error bars indicate standard deviation of V^*_{max} calculated from the Michaelis–Menten kinetic curve with 11 data points.

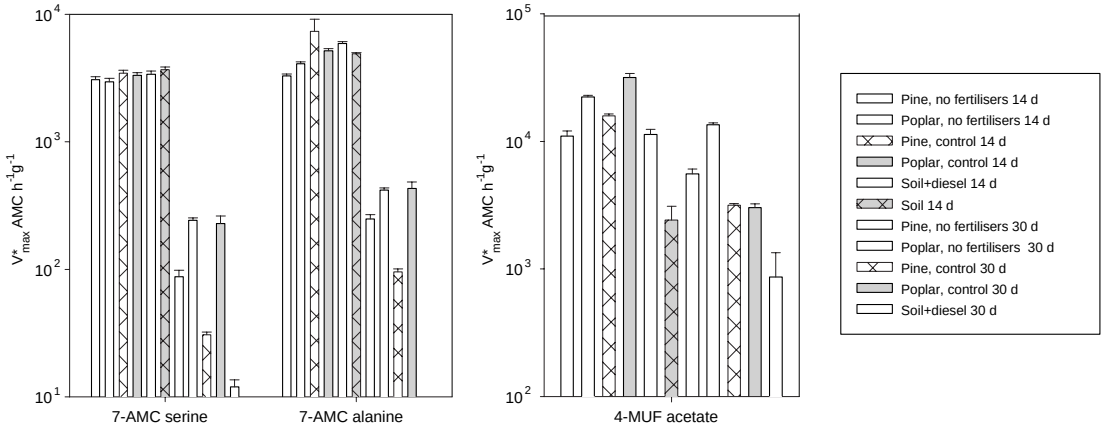

Figure 5. Potential maximum rate of enzymatic activity V^*_{max} of serine and alanine aminopeptidases (μM AMC/h/g dry soil) and acetate esterase (μM MUF/h/g dry soil). Activities measured in pine and poplar vegetated soil and control soil after the second diesel fuel addition. Error bars indicate standard deviation of V^*_{max} calculated from the Michaelis–Menten kinetic curve with 11 data points.

1.4×10^6 to 8.4×10^7 cfu/g dry soil. The results indicate that vegetation and soil amendments did not strongly affect the viable plate counts on soil agar.

Comparison of the utilised assays

The, 2–3 days incubation time with Biolog plates, was sufficient to follow up the utilisation of organic substrates by soil microbiota. Enzyme assays require much shorter incubation times and report biocatalytic activities that correspond more to *in situ* field conditions. Furthermore, soil enzyme activity analysis provides information about cleaving rates of different chemical bonds in soil biopolymers, while the Biolog method measures metabolic activity of the organic substrate utilisation of soil bacteria that can tolerate high substrate concentrations. Both methods were used to describe the capability of the microbial communities to respond to changing environmental impacts, phytoremediation of an oil spill removal in the concert with phytoremediation.

Diesel fuel utilisation assays done with MT2 plates detected biological responses to diesel addition better than viable counts, MEE activities or carbon source utilisation patterns. General physiological activity tests are secondary to con-

taminant biodegradation assays when assessing bioremediation potential. Thus, general microbial activity assays alone should not be used to compare bioremediation treatments.

Conclusions

Hydrocarbon contamination resulted in minor changes in carbon source utilisation patterns and increased the ability of soil microorganisms to utilise diesel fuel. The activities of extracellular esterases and aminopeptidases increased after diesel fuel addition, but started to decrease with time. Our applied methods, except viable counts, were able to detect the response in microbial activity after diesel fuel addition and may be useful bioassays to monitor and study oil-spill impacts, whereas the changes with general microbial activity methods were less pronounced. The assay with diesel utilisation in MT2 plate was the most significant parameter.

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