ASSESSMENT OF MICROBIAL COMMUNITY CHANGES AND LIMITING FACTORS DURING BIOREMEDIATION OF HYDROCARBON-POLLUTED SOIL WITH NEW MINIATURIZED PHYSIOLOGICAL METHODS

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Summary

Due to human activities, organic pollutants are spilled to the environment where they threaten public health, often as contaminants of soil or groundwater. Living organisms are able to transform or mineralize many organic pollutants, and bioremediation techniques have been developed to remove pollutants from a contaminated site. However, fast and easy methods to document both the efficacy of bioremediation and the changes in soil microbial communities during bioremediation are not well developed. The major aim of this thesis was to develop miniaturized methods targeting the physiology of microbial communities during pollutant degradation in soil and to assess pollution-induced community changes. Furthermore, the methods should identify factors limiting efficient pollutant biodegradation. Petroleum hydrocarbons have been chosen as pollutants because these compounds are frequently spilled, readily water-soluble, partly toxic and carcinogenic, and therefore undesirable in soils and drinking water supplies.

The influence of petroleum hydrocarbons on a microbial community in the vadose zone was assessed under field conditions. An artificial hydrocarbon mixture consisting of volatile and semi-volatile compounds similar to jet-fuel was emplaced in a previously uncontaminated vadose zone in nutrient-poor glacial melt water sand. The experiment included monitoring of microbial parameters and CO_2 concentrations in soil gas over 3 months, in- and outside the hydrocarbon vapor plume that formed around the buried petroleum. Microbial and chemical analyses of vadose zone samples were performed on material from 9 cores drilled on 3 days at 3 distances from the buried petroleum mass to 3.3 m depth. Significantly elevated CO_2 concentrations were observed after contamination. Total cell numbers as determined by fluorescence microscopy were strongly correlated with soil organic carbon and nitrogen content but varied little with contamination. Redundancy analysis (RDA) allowed direct analysis of effects of selected environmental variables or the artificial contamination on microbiological parameters. Variation in biomass and CO_2 production was to 46 % explained by soil parameters and to 39.8 % by the duration of contamination. The microbial community structure was assessed by community-level physiological profile analysis using Biolog™ EcoPlates. Only 35.9 % of variation in Biolog™ data could be attributed to soil parameters and contamination, however, the samples with greatest exposure to hydrocarbons grouped together on RDA plots. It is concluded that at this nutrient-poor site, the microbial community was dominated by the natural heterogeneity and that the influence of petroleum hydrocarbon vapor was weak.

Biolog™ Eco-Plates combined with RDA were able to distinguish between samples with high and low hydrocarbon exposure. However, the method was not sensitive enough to

produce consistent patterns in three replicates when extracts from subsurface soils with low cell numbers were inoculated. A more sensitive, MPN-based system was therefore developed and its ability to provide information about specific pollutant degraders was tested in alluvial sand. Eight dilutions of sand extracts were inoculated on medium- and substrate-containing polypropylene deep-well plates. To keep the concentrations of the volatile substrates constant over several days of incubation without intoxicating the soil community, a substrate-containing organic carrier phase was applied to each vial. The biochemically inert 2,2,4,4,6,8,8-heptamethylnonane (HMN) was appropriate for this purpose. Thus, the volatile substrates partition from HMN into the microorganism-containing soil water where they achieve relatively low concentrations. The sensitive water-soluble tetrazolium dye (WST-1) was added to each well after 7 days incubation to detect dehydrogenase activity. A dark yellow signal was developed for the substrates toluene, *n*-octane, *n*-dodecane, 1,2,4-trimethylbenzene and methylcyclohexane but not for isooctane, being strongest in *n*-alkane amended wells. With this method, more hydrocarbon degrading bacteria were detected in an alluvial sand exposed to kerosene for 72 hours compared to the community in the pristine sand. Both communities were mainly composed of *n*-alkane degraders. Although encouraging results were achieved, we noticed that the tetrazolium reduction was inhibited in some cases. Wells with high cell numbers, in which obvious growth occurred, did not produce a signal.

In a next step, the two-liquid phase system with HMN was applied to modify a miniaturized respiration detection system using whole soil samples instead of soil extracts (Campbell *et al*., AEM 69, p. 3593). Soil was incubated in deep-well plates in presence of single volatile organic hydrocarbons. The carrier phase was added to the interstitial space in a bead layer on the bottom of the well (below the soil sample), and the hydrocarbons diffused into the soil-water. The soil activity was determined by means of an agar plate containing a pH indicator, which changes color as a function of the produced CO_2. Physiological profiles specific to petroleum hydrocarbons in pristine and contaminated soils were assessed. The substrate concentrations, which induced highest microbial activity, as well as concentrations causing inhibition, were determined with effect-concentration curves. Community-level physiological profiles based on hydrocarbon degradation were obtained by applying multivariate analysis on the CO_2 yields. The first measurement period of 0-6 hours after addition of relatively high hydrocarbon concentrations to the system yields values, which best separated soil types as well as pristine and contaminated soils. If low concentrations were applied, we obtained only significant separations after 24 hours incubation, probably after a growth step.

The same micro-respiration system equipped with a $^{14}CO_2$ detection plate was applied to study N and P limitation of aerobic mineralization of ^{14}C-toluene in a soil previously ex-

posed to petroleum. Significant nutrient limitation during short-term tests (24-48 hours) was identified and the optimized combination of nutrient addition was determined.

Conclusively, miniaturized physiological methods have been proven to detect soil microbial community changes in petroleum hydrocarbon-contaminated soils. Adding an organic carrier phase to the miniaturized systems allowed assuring a volatile organic hydrocarbon supply in non-toxic concentrations in the soil water or the medium for several days. Thus simple, cost-effective systems to investigate the soil community during bioremediation and to address questions of nutrient shortage in soils have been provided with this thesis.

Version abrégée

La contaminantion du sol et des nappes phréatiques par des polluants organiques liée aux activités humaines met en danger la santé publique. Certaines souches microbiennes capables de transformer ou de minéraliser des polluants organiques sont à l'origine du développement des techniques de bioremédiation pour la dépollution des sites contaminés. Cependant des méthodes simples et rapides à mettre en oeuvre pour évaluer l'efficacité de la bioremédiation et les changements dans la communauté bactérienne dus à la bioremédiation sont rares. Le but principal de cette thèse était de développer une méthode physiologique miniaturisée afin d'évaluer les changements de la communauté bactérienne suite à la bioremédiation. De plus, la méthode devrait aussi permettre de détecter des facteurs limitants l'efficacité de la biodégradation. Des hydrocarbures de pétrole ont été choisis comme polluants dans cette recherche parce qu'ils sont fréquemment déversés dans l'environnement, bien qu'en raison de leurs bonnes solubilités dans l'eau, de leur toxicité ou du fait qu'ils soient carcinogènes, ces polluants sont indésirables dans l'eau.

L'influence des hydrocarbures de pétrole sur la communauté bactérienne dans la zone non saturée a été étudiée sur le terrain. Un mélange artificiel d'hydrocarbures volatiles et semivolatiles, semblable au kérosène, a été introduit dans une zone auparavant non contaminée. Le sol était un sable formé par de l'eau de fonte de glace pauvre en nutriments. L'expérience a consisté à mesurer pendant trois mois les paramètres microbiens et les concentrations de CO_2 dans l'air du sol, à l'intérieur et à l'extérieur du panache, qui s'est formé autour de la source d'hydrocarbures enfouie dans le sol. Des analyses microbiologiques et chimiques du sol ont été réalisées sur neuf carottes prélevées à trois dates, trois distances différentes de la source et à une profondeur de 3.3 m. Les concentrations de CO_2 ont augmenté significativement après la contamination. Le nombre des cellules, déterminé par microscopie à fluorescence, était fortement corrélé avec le taux de matière organique et la quantité d'azote total, mais variaient peu avec la contamination. L' analyse de redondance (redundancy analysis) (RDA) a permis de lier les effets des variables environnementales ou de la contamination artificielle avec les paramètres microbiens. La variation de biomasse et de production de CO_2 était expliquée à 46 % par les paramètres du sol et à 39.8 % par la durée de la contamination. La structure de la communauté microbienne dans ce sol a été déterminée par l'analyse des profils physiologiques au niveau de la communauté (CLPP) en utilisant des plaques Biolog™ (EcoPlates). Seuls 35.9 % de la variation des CLPPs pouvaient être attribués aux paramètres du sol et à la contamination; cependant les échantillons qui ont été exposés aux plus grandes concentrations de polluants étaient regroupés par la RDA. Il en a été conclu que la communauté microbienne

de ce site pauvre en nutriments est principalement influencée par l'hétérogénéité naturelle du sol et que l'influence des vapeurs d'hydrocarbures de pétrole est faible.

L'utilisation de plaques Biolog^{TM} combinée avec la RDA a permis de différencier les échantillons qui ont été exposés aux hautes concentrations d'hydrocarbures de ceux qui ont été exposés à des concentrations basses. Pourtant la méthode n'était pas assez sensible pour produire trois replicats cohérents lorsque des extraits du sol de la subsurface, contenant peu de microorganismes, étaient inoculés sur les plaques. Un procédé plus sensible basé sur la méthode du nombre le plus probable (MPN) a été développé, afin d'obtenir des informations spécifiques sur le nombre de microorganismes capables de dégrader certaines polluants dans un sable alluvial. Un extrait du sable a été inoculé sur une plaque de polypropylène aux puits profonds contenant un milieu de croissance et un substrat. Une phase organique, le 2,2,4,4,6,8,8-heptaméthylnonane (HMN) substance biochimiquement inerte, servant de réservoir a ensuite été ajoutée à chaque puits afin de conserver la concentration des substrats volatils constante pendant plusieurs jours sans intoxiquer les microorganismes. Les substrats volatiles diffusent de l'HMN vers la phase aqueuse contenant les microorganismes, où ceux-ci sont exposés à des concentrations d'hydrocarbures relativement basses. Un tetrazolium sensitive (WST-1), soluble dans l'eau, a été ajouté dans chaque puits après sept jours d'incubation pour détecter l'activité des deshydrogènases. Un signal a été développé dans tous les puits sauf dans ceux ayant comme substrat l'isooctane. Le signal le plus fort a été produit par les microorganisms croissant sur des *n*-alcanes. Cette méthode a permis de mesurer une augmentation du nombre de bactéries capables de dégrader des hydrocarbures dans le sable contaminé par rapport au sable non-contaminé. Les communautés bactériennes des deux sables étaient dominées par des organismes dégradant les *n*-alcanes. Même si les résultats obtenus avec la méthode WST-MPN étaient encourageants, il a été noté que la réduction du tetrazolium était inhibée dans certains cas. Les puits dans lesquels une croissance a pu être observée n'ont pas produit de signal.

Dans l'étape suivante, la technique du réservoir organique (HMN) a été appliquée dans un système de micro-respiration, dans lequel des échantillons de sol entier sont utilisés à la place d'extraits de sol (Campbell *et al.*, AEM 69 p. 3593). A nouveau, le sol a été incubé dans des plaques aux puits profonds en présence d'un seul hydrocarbure volatile organique, à la différence que le réservoir organique a été pipeté dans l'espace interstitiel d'une couche de billes placées au fond de chaque puit. Dès ce moment-là, le composé organique a diffusé dans la solution du sol. Dans ce système l'activité microbienne du sol peut être mesurée avec un agar auquel un indicateur pH détectant la production du CO_2 a été ajouté. Des profils physiologiques spécifiques aux hydrocarbures ont été déterminés dans des sols contaminés et non-contaminés. Avec des courbes d'effets des substances en fonction

de la concentration, les concentrations qui induisaient la plus grande activité microbienne et celles qui causaient une inhibition ont pu être mises en évidence. Des CLPP ont été obtenus en appliquant une analyse multivariée aux concentrations de CO_2 produits lors de la dégradation des substrats. Les mesures sur la première période de 0-6 heures après l'ajout des concentrations élevées d'hydrocarbures dans les puits ont montré la meilleure séparation entre les différents types du sol et entre les sols contaminés et non-contaminés. Lorsque des concentrations basses d'hydrocarbures ont été appliquées, les types de sols ne pouvaient être distingués uniquement après 24 heures d'incubation, qui correspond probablement à une phase de croissance des microorganismes.

Le même système de micro-respiration, cette fois-ci équipé d'une plaque de détection du $^{14}CO_2$, a été mis en oeuvre afin d'étudier les limitations liées à P et N de la minéralisation en aérobie de ^{14}C-toluène dans un sol contaminé. Des limitations dues aux nutriments ont été identifiées pendant des tests de courte durée et une combinaison de nutriments optimale pour ce sol a pu être déterminée.

En conclusion, il a été prouvé que des méthodes physiologiques miniaturisées sont des outils performants de détection des changements survenant dans les communautés microbiennes des sols contaminés par des hydrocarbures de pétrole. L'ajout d'un réservoir organique dans le système a permis d'assurer un approvisionnement en hydrocarbures dans la solution du sol en concentrations non-toxiques pendant quelques jours. Cette thèse a permis de mettre en place des systèmes simples et rentables afin d'étudier la communauté bactérienne durant la bioremédiation, permettant également de répondre aux questions concernant les limitations nutritives dans les sols.

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CHAPTER 1

General Introduction

Developed countries including Switzerland are challenged by significant damage to the environment by a multitude of contaminants as a consequence of industrial production (3). Biodegradation of individual organic chemicals has been a field of research for around 50 years (6). The initial interest was in the fate and persistence of pesticides in soils; however, the field has expanded enormously to include a wide variety of substances. A significant factor in the development of remediation technology has been the enactment of environmental laws and regulations which favour waste treatment to waste disposal *e.g.* in landfills. Biological treatment methods have often been found to be less expensive than chemical or physical methods (57). The technologies that have been developed considerably enhance biodegradation and result in microbial destruction of organic pollutants that otherwise would persist at the sites of contamination (6). However, biological treatment is not the most suitable in all cases. The optimal strategy for any given site will depend on the nature and concentration of the contaminants, the hydrogeology of the site, and the extent of contamination (3). This chapter provides insight into the requirements of microorganisms for efficient biodegradation of volatile petroleum hydrocarbons and their interactions with soils and pollutants.

1.1 Contamination and clean-up in Switzerland

Groundwater is the main drinking water resource in Switzerland. By the end of the eighties it was increasingly found that the groundwater was contaminated with toxic substances such as perchloroethene (PCE) and mineral oil compounds, so that pumping stations

had to be closed (91, 92). It took another few years until the *Contaminated Sites Ordinance (CSO)* (40), based on the environmental protection law, was enacted in 1998. According to this ordinance, authorities of each canton are obliged to elaborate a cadastre on polluted locations. A nationwide database containing an estimated number of 50'000 entries was supposed to be completed by the end of 2003. These entries will be further investigated and ranked in contamination priorities. Estimated 3000-4000 polluted locations will figure on the first priority list. They are threatening human health and the environment and are thus classified as contaminated sites. According to the ordinance they have to be remediated within the next 25 years (one generation), causing estimated costs of 5 billion CHF or about 4 billion USD (92). By definition, a polluted area becomes only a contaminated site if life-sustaining goods are in danger. Hence, the *CSO* primarily protects soil fertility of agri- and horticultural land, ground- and surface waters, and public areas such as children's playgrounds. In the same year as the *CSO,* a revised form of the *Ordinance Relating to Impacts on the Soil (OIS)* (120) was enacted. The *OIS* protects by definition the plant growth-supporting surface soil, whereas the subsoil is excluded from this ordinance. The *OIS* does not contain any guide, trigger or clean up values for many chemicals such as volatile organic carbons (VOCs). Thus, the subsoil is only protected by the *CSO* and only if life-sustaining goods are endangered. Maximum concentrations in leachate as well as pore air concentrations for BTEX (benzene, toluene, ethylbenzene, xylenes) and various other organic and inorganic compounds are defined in the *CSO*.

1.2 Soil and vadose zone

Hydrogeologists and researchers involved in contaminated site issues often use the term vadose zone instead of soil. It means 'the geologic media between land surface and the regional water table' (145) and includes the soil zone, the intermediate vadose zone (unsaturated zone) and the capillary fringe (104) (Figure 1.1). The vadose zone is typically the first subsurface environment encountered by contaminants after a terrestrial spill. As a result, all subsequent groundwater and surface water contamination and any resulting environmental impact, are influenced by the contaminant behaviour in this dynamic system (104). The soil zone often exhibits weathered soil horizons and provides mechanical support and nutrients for plant growth. A broad range of microorganisms is nearly always present in the vadose zone although population densities vary widely (5, 53). The surface of soil particles is the site where many of the biochemical reactions in the cycling of organic matter, nitrogen, and other minerals, in the weathering of rocks and in the nutrition of plants and microorganisms take place (7). Throughout the vadose zone, the pores and fractures are partially filled with water. Only in the capillary fringe completely filled pores are found. We had the opportunity to investigate and relate microbial community

changes, soil properties and petroleum hydrocarbon concentration throughout the vadose zone to the aquifer on the occasion of a large field experiment (Chapter 2).

1.3 Organic contaminants

Organic compounds found in soils can be classified as naturally occurring or of anthropogenic origin. Naturally occurring organics resulting from the decay of plant and animal tissues, termed humic materials, are normal constituents of soils and groundwaters. Humic materials are structurally complex and are very resistant to further biological transformation. Organics of anthropogenic origin range widely in characteristics (57) and comprise pesticides, solvents, petroleum hydrocarbons, etc.. Anthropogenic compounds have affected nearly every natural ecosystem in the world. What is a contamination if natural organics are recalcitrant and anthropogenic compounds ubiquitous? A commonly held view is that contamination occurs when the soil composition and function deviate from the normal composition and function (4).

Petroleum hydrocarbons are prevalent soil pollutants (33). Petroleum is an extremely complex mixture of hydrocarbons. From the hundreds of individual components, several classes based on related structures can be recognised: an aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction. This work focuses on the volatile non-polar fraction. Properties of these compounds with particular significance for bioremediation are polarity/solubility, volatility, toxicity and biodegradability (57).

Polarity/solubility: Non-polar compounds tend to be water-immiscible and partition into the organic materials found in vadose zones. The result is that they are generally less mobile in soils and groundwater, and the spreading of non-polar compounds in groundwater and aquifers is generally slower than the spreading of polar compounds. Contaminants must be in solution for biodegradation processes to occur since the aqueous phase is the microorganism's habitat which provides them with nutrients (57) (Figure 1.2). Hence, the solubility and the partitioning rate from the organic to the aqueous phase have a high impact on biodegradation rates.

Volatility: Volatile compounds tend to partition from the petroleum phase into the gaseous phase. They are often quite mobile in unsaturated soils, and emissions from the vadose zone may constitute a hazard at the contamination site or during excavation (57, 129).

Toxicity: The key factor driving the need for remediation of contaminated soil and groundwater is human and environmental toxicity. Disposal or discharge of toxic chemicals to soils presents a difficult problem because 1) toxic materials may be resistant to bio-

degradation, 2) once materials are in the soil environment less control exists with respect to their transport and fate, and 3) the risk to water supplies is very high because many substrates induce toxic effects in low concentrations (57). A method, which allows estimating inhibiting hydrocarbon concentrations towards the soil microbial community, is applied in Chapter 4.

Biodegradability: Readily metabolizable, non-toxic (to the microorganisms) organic compounds are normally very rapidly oxidized in the vadose zone. Biodegradability is related to factors such as solubility, the degree of branching, the degree of saturation, and the nature and extent of substitution (57). The effect of branching is seen in the relative degradability of isomers (65, 136). *n*-Octane for example is more easily degraded than isooctane (2,2,4-trimethylpentane) although both have the stoechiometric formula C_8H_{18} (see Table 1.1) (123) and much less strains are able to grow on 2,2,4,4,6,8,8-heptamethylnonane (HMN) than on hexadecane, a fact we exploit with the experimental system presented in this work.

1.3.1 Origin of contaminations

Serious soil and groundwater contamination problems may result from accidental spills and improper disposal of toxic materials. Accidental spills during transports of chemicals may result in large quantities of pure products on small areas of ground. Disposal of chemicals in homes and enterprises is and was often done in a manner that resulted in soil and groundwater contamination. Waste oil and cleaning solvents from garages, agricultural chemical residuals on farms and at airfields used by crop duster paints, and cleaning supplies have often been dumped onto soil or buried on private land (57).

The most ubiquitous soil and groundwater contamination in the world is undoubtedly gasoline leakage from underground storage tanks (33, 57). Among the most commonly encountered contaminants in soils and groundwater are aromatic hydrocarbons such as BTEX, resulting from spills or leakage, and chlorinated aliphatics such as perchloroethene (PCE) or trichloroethene (TCE), used in industry for degreasing (111). Contaminant plumes tend to travel slowly in the unsaturated (vadose) zone but will eventually reach groundwater if leakage continues. Mixing with water in the aquifer is a function of relative density and miscibility. Water immiscible compounds are called non-aqueous phase liquids (NAPL). Light NAPLs tend to float (petroleum hydrocarbons), dense NAPLs tend to sink (PCE, TCE). Cases of gasoline and jet fuel leakage have often resulted in pools of free product accumulating over an unconfined aquifer (57), forming a plume as illustrated in Figure 1.1.

Today a wide assortment of petroleum-derived compounds is used as pesticides and herbicides. Petroleum-based chemicals used in modern society are staggering: plastics, pharmaceuticals, and detergents. Many compounds, in some cases after extensive world-wide use, have found to be toxic for wildlife and fish (*e.g.* DDT), highly toxic to mammals (*e.g.* Parathion) or carcinogenic (*e.g.* PCB, benzene) (58). The complexity of the ecosystem makes the determination of the effects of chemical use very difficult. To complicate the matter of the enormous amounts of organic contaminants released into the environment, we should not forget metabolites that are produced during degradation. A metabolite on the anaerobic degradation pathway of PCE or TCE to ethene and finally CO_2 is vinylchloride (VC). This compound is much more toxic and carcinogenic than TCE or PCE (167).

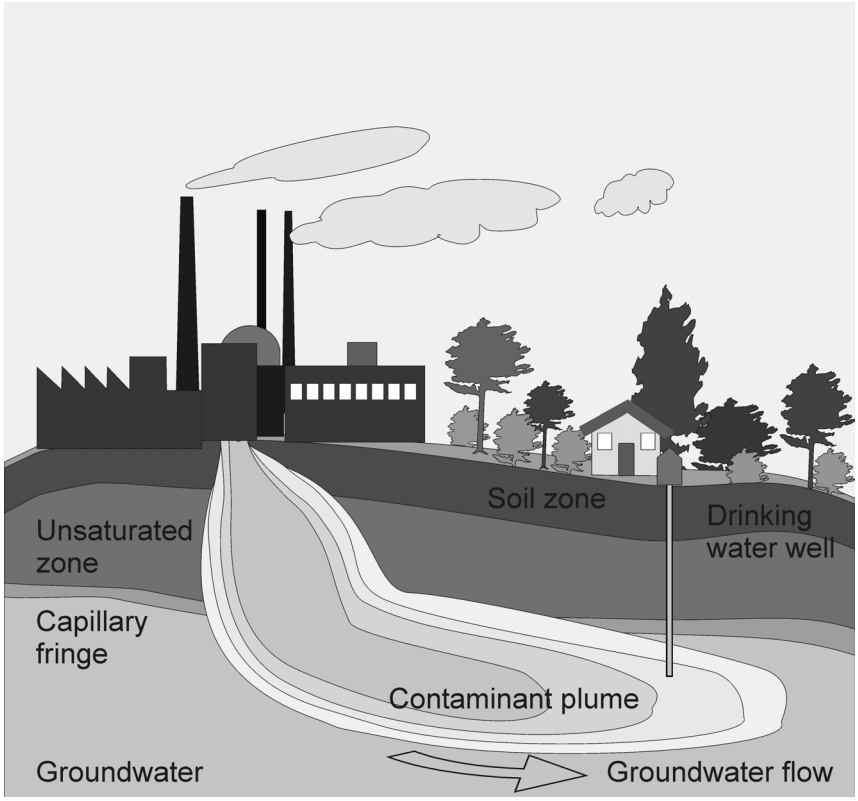

Figure 1.1: Schematic diagram showing a groundwater resource, which is protected by the vadose zone consisting of the soil zone, the unsaturated zone and the capillary fringe. The contaminant plume migrates in the direction of the groundwater flow.

1.4 Vadose zone and organic contaminants

The vadose zone is an environment that is composed of heterogeneous matrices that are combinations of solid, liquid, and gaseous phases. Any chemical existing in such an en-

vironment is subject to the laws of chemical equilibrium that govern the partitioning of the compound into the various phases of the system (48) (Figure 1.2), the solid soil phase, the water phase and the gaseous phase. The mass fraction of the compound in each phase is an indication for transport and biological and chemical processes going on in the vadose zone.

The partitioning of a chemical between the water and a non-aqueous phase liquid (NAPL) is based on its solubility in each of them. This relative partitioning can be expressed by *e.g.* the octanol-water coefficient K_{OW}, for which extended databases are available. A high K_{OW} indicates that a compound is hydrophobic and that little of it would be present in water that is in equilibrium with NAPLs. A compound with a low K_{OW} would exist at higher concentrations in water. Direct partitioning between NAPL and water can be calculated with Raoult's law based on mole fraction of the volatile compound in the NAPL and the water-solubility. It has highest significance in soils with high water content or aquifers.

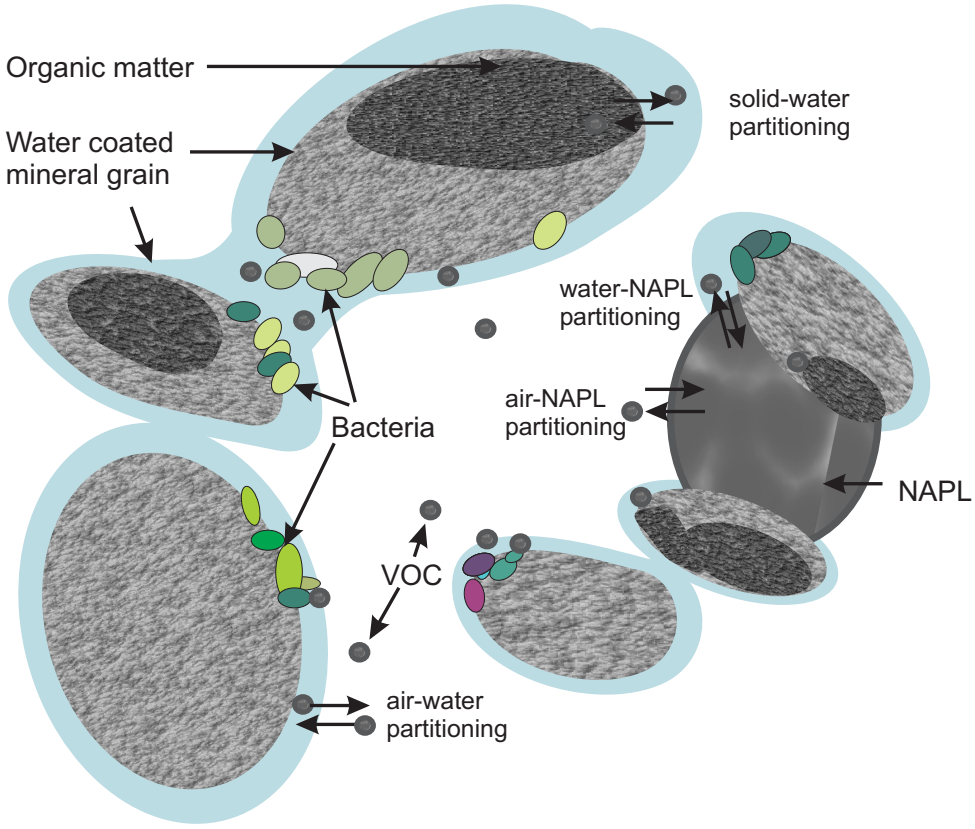

Figure 1.2: Partitioning of volatile organic compounds (VOCs) between the main soil phases. Modified from Werner (164).

Soil moisture is important in determining the extent of adsorption of neutral, non-polar molecules, which are strongly adsorbed to soils at low moisture content (124). They are displaced as soon as soil moisture increases as a result of competition for adsorption sites. The higher the soil water content, the more pore space is filled with water, and the more flow paths become restricted. Soil organic matter adsorbs non-polar compounds. Its similarity to the NAPL is expressed in the highly positive correlation between partitioning coefficients K_{OW} and K_{OC} (139). K_{OC} is a measure of the partitioning of a compound between an aqueous phase and a phase with high organic carbon content such as humus.

Volatilization includes the loss from chemicals from a surface into the vapour phase. This process includes vaporization or partitioning from a NAPL, the soil matrix or the water phase into the gaseous phase and hence the atmosphere. Henry's law constant H describes the air-water partitioning.

We will use this partitioning property of non-polar compounds in the following studies. We apply HMN as NAPL in the function of a carrier phase. HMN is a compound that is chemically more closely related to hexadecane than to octanol and therefore we will use $K_{Hex\text{-}Water}$ instead of K_{OW}. Information that is more detailed will be found in the Appendix I.

1.5 Bacteria

Bioremediation is carried out by microorganisms, principally bacteria and fungi. Microorganisms decompose organic and inorganic compounds, either pollutants or natural substrates, to obtain carbon and/or energy for growth or maintenance of their metabolism (57). The substrate oxidation process in bacteria releases electrons that pass through a chain of reactions within the cell and which in the end must be discharged to the environment. We basically distinguish bacteria, which live in oxic or anoxic environments. Common in organisms with aerobic biodegradation is that the major degradative pathways for hydrocarbons involve oxygenases and molecular oxygen (13). The terminal electron acceptor O_2 is the final recipient of the electrons which is discarded as H_2O (57). If substrate molecules are oxidized bacteria release metabolites or CO_2. O_2 depletion as well as metabolite or CO_2 increase in the environment are indicators of aerobic microbial activity. We benefit from CO_2 as activity indicator in all the following studies, but it is particularly important in Chapters 4 and 5. Further essential needs, except a substrate electron donor and acceptors of microorganisms, are nutrients, trace-metals, and water.

1.6 Bacteria and soil

1.6.1 Microbial abundance and distribution

A surface soil microbial community has been estimated to consist of 10'000 different species and between 10^7 and 10^{10} individual cells g^{-1} soil (154) depending on soil type and depth. Amann *et al.* (10) estimated that about 1 to 5 % of the organisms living in soil can be cultivated on common enrichment media, but Torsvik *et al.* (156) found only 0.59 % isolates in comparison to the estimate of species obtained by molecular biological methods. Microbes are not equally distributed throughout the whole soil volume but tend to concentrate in the rhizosphere, or in plant and animal organic debris (72) where they form microcolonies (156). Microbial abundance in the vadose zone varies with depth concomitant with a lower nutrient flux in the intermediate vadose zone than in aquifers or surface soils (5, 9). Differences in water and nutrient flux in flow bypass compared to preferential flow regions generates heterogeneity in microbial populations and activity (5).

1.6.2 Diversity

It has been acknowledged that the wealth of genetic complexity within soil by far exceeds that of any other habitat on earth (154, 163). High biodiversity is beneficial to ecosystem performance since it guarantees a certain functional redundancy and enhances productivity, stability and resilience (117, 118). Consequently in a system of low diversity, any applied stress such as pollution or agricultural management (156) or perturbations removing species may cause a total loss of function, whereas in a highly diverse system species loss will have little impact (18). The biodiversity, which can also be regarded as the amount of information available in a community, is considered an important parameter to describe the multifunctionality and flexibility of a soil (156). Knowledge about community composition and functioning is essential to understand the performance and responsiveness in changing environments such as terrestrial systems during pollution and remediation. Therefore, it is essential to have knowledge of microbial community function and functional diversity (125).

1.7 Bacteria and petroleum hydrocarbons

1.7.1 Effect of stress due to contamination

An effect on the microbial community in soils is observed if an impact (*e.g.* a contamination) is of significance for the competition, performance, reproduction, or survival of any of the community members. An effect on the community should not be considered an all-

or-none phenomenon, but rather a quantitative one dependent on preexposure time and concentration (144). An impact of ecological relevance will eliminate or hinder the success of sensitive species, favour more tolerant ones and cause a toxicant-induced succession that can be observed as a change in community structure (21). Therefore good indication of community changes due to contamination is the absence of sensitive species. This measurable process is termed pollutant induced community tolerance (PICT) and a good indicator for the pollution history of an area (23).

A large number of microcosm studies have demonstrated sizable increases in populations of hydrocarbon-utilizing microorganisms when environmental samples were exposed to petroleum hydrocarbons (79, 142). MacNaughton *et al.* (105) observed a community shift in petroleum contaminated soil from gram-positive to gram-negative bacteria with the rare *α-Proteobacteria* becoming abundant. In general, population levels of hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons. Atlas (13) found that in unpolluted ecosystems, hydrocarbon utilizers generally constitute less than 0.1 % of the microbial community; in oil-polluted ecosystems, they can constitute up to 100 % of the viable microorganisms. There are three ways by which adaptation upon exposure of the population to a new substrate can occur: 1) Induction or derepression of specific enzymes not present (or present at low levels) in the population before exposure, 2) selection of new metabolic capabilities produced by genetic changes, and 3) increase in the number or organisms able to catalyze a particular transformation. The third type of change often follows one of the first two (144).

1.7.2 Biodegradation

Microorganisms transform contaminants through metabolic reactions. The processes of interest in this work involve biooxidation of organic contaminants. Two types of biodegradation of organic compounds may occur - mineralization or biotransformation. Biotransformation is an incomplete biodegradation, which may transform a compound into a stable organic metabolite, which can be innocuous or less toxic. However, also the opposite, a so-called activation, may occur as we have seen in the example of vinyl chloride earlier. The phenomenon of biotransformation often accompanies co-metabolism, the biodegradation of organic compounds from which no carbon or energy is derived for microbial metabolism (6, 63). Co-metabolism of an organic pollutant typically occurs at the same time as the utilization of a primary substrate (48). Mineralisation occurs when there is complete biodegradation of an organic molecule to inorganic compounds, *e.g.* water, carbon dioxide, mineral ions (based on nitrogen, phosphorous, sulphur, etc.) (6). During transformation electrons are transferred from these organic compounds to electron accep-

tors and the carbon skeleton is oxidised. The energy released is used for synthesis of new cell material, for repair of damaged cell parts, transport of compounds into the cell or movement (57).

The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera; a diverse group of bacteria, fungi and algae have been shown to have this ability (13). ZoBell (177) recognized that such microorganisms are widely distributed in nature. However, finding indigenous subsurface microorganisms that degrade the more recalcitrant compounds, such as polycyclic aromatic and highly chlorinated compounds is more difficult (151).

Metabolic pathways have been established for the degradation of a number of compounds. There are most often several microbes able to degrade one and the same compound under different environmental conditions. It does not astonish that different degradation pathways have developed. For example, toluene can be degraded in at least five different ways under aerobic conditions (174).

1.7.3 Biodegradation of compounds in NAPLs

NAPLs affect the concentration of contaminants available for degradation by organisms. A NAPL may sequester a large fraction of a (volatile) hydrophobic pollutant away from the aqueous phase. If the aqueous concentrations of NAPL contaminants are high, rates of mineralization are higher than the rates of partitioning of a contaminant to water (51). With low NAPL concentrations, biodegradation rates are slower than at higher concentrations and much slower than partitioning (129). This may result in unexpectedly low biodegradation rates or in contaminant concentrations below the threshold for biodegradation (129).

Biodegradation rates in kerosene hydrocarbon contaminated soil (Værløse) and alluvial sand are given in Table 1.1. The same alluvial sand was used in Chapter 3. Chapter 2 describes community changes in the Værløse soil before and after kerosene contamination. Table 1.1 shows that *n*-alkanes with chain lengths >8 carbons and the aromatic hydrocarbons toluene, m-xylene, and 1,2,4-trimethylbenzene are degraded at the highest rates. Cyclic alkanes and *n*-hexane undergo degradation at slower rates, whereas for *n*-pentane, isooctane, and methyl-*tert*-butyl ether (MTBE) degradation is slow.

1.8 Soil, organic contaminants and bacteria: Remediation

A broad range of technologies is available for the clean up of contaminated sites. The remediation approach selected for a given contamination should be site and incident specif-

ic. They are basically categorised in physical, chemical and biological methods and subdivided in 'Off site' and 'On site' treatments. In most cases several approaches are combined (129).

Table 1.1: Biodegradation rates estimated at different experimental scales during the GRACOS project (see Chapter 2 and (67)). Rates are given as apparent first order rates applied to the gaseous phase, in d^{-1}. See Refs. (79, 123) for definitions. For conversion to aqueous phase rates see equation 5 in Ref. (123).

	Alluvial Sand from Lake Geneva [a]			Værløse Soil from 1 m depth [b]	
Compound	Column [c, j]	Lysimeter [d, k]	Batch tests [e, k]	Column [f, l]	Field [g, m] (MIN3D)
n-Pentane	<0.01	<0.05	b. d. l	n.d.	n.d.
n-Hexane	0.26	0.4	0.65±0.35	0.10±0.04	0.004
n-Octane	5.0	6.7±1.7	10.4±2.8	1.23±0.2	1.08
n-Decane	13.5	5	b. d. l	5.83±1.1	3.53
Cyclohexane	0.07	0.5±0.3	b. d. l	n.d.	n.d.
Methylcyclohexane	0.16	0.8±0.4	b. d. l	0.31±0.08	0.10
3-Methylpentane	n.d.	n.d.	n.d.	0.06±0.02	0.01
Isooctane[h]	0.09	0.12±0.03	b. d. l	0.20±0.01	0.01
Benzene	n.d.	n.d.	n.d.	0.21±0.12	1.95
Toluene	1.31	3.2	1.95±0.53	0.70±0.2	0.27
m-Xylene	3.28	n.d.	3.68±1.21	1.65±0.6	1.65
1,2,4-Trimethylbenzene	4.98	n.d.	b. d. l	3.70±0.4	2.56
MTBE[i]	<0.01	<0.05	b. d. l	n.d.	n.d.

[a] Nutrient-rich sand, with parameters described in Ref. (123). [b] Glacial melt-water sand, low in nutrients, with parameters described in Ref. (97). [c] Soil water content θ_w=0.118, Temperature T= 23±2°C. [d] θ_w=0.05, T= 18±2°C. [e] θ_w=0.05-0.13, T= 25°C, batch data affected by non-steady state sorption (79). [f] θ_w=0.094, T= 25±2°C. [g] inverse modeling using MIN3D. [h] 2,2,4-Trimethylpentane, [i] Methyl *tert*-butyl ether, [j] Ref. (79), [k] Ref. (123), [l] Ref. (97), [m] Ref. (106), b.d.l. below detection limit, n.d. not determined

1.8.1 Bioremediation

Bioremediation is a very cost-effective and sound remediation method (129), equally successful in reducing the concentrations of single compounds or mixtures of biodegradable materials (100). Bioremediation can make use of four features of an active population: their need for assimilatory nutrients such as C, N, P, S, K, etc., their need for a source of electrons, their enzymatic non-specificity (co-oxidation), and their need for terminal electron acceptors (157). Environmental constraints which affect degradation are molecular oxygen, pH, temperature, oxidation-reduction potential, availability of inorganic nutrients (*e.g.* nitrogen and phosphorous), salinity, the concentration and nature of the organics (129) and presence of inhibitory or toxic agents (2). If microbial activity stops (*e.g.* due

to build up of toxic metabolites), restarting the process may be extremely difficult. Success of a bioremediation project is dependent on the ability of process operators to create and maintain environmental conditions necessary for microbial growth (57). Disadvantages of bioremediation are the difficulty in predicting the performance, the scaling up from laboratory tests, and of course, the recalcitrance of certain substances. Bioremediation methods have been applied successfully in restoring polluted sea shores, airports, military operation and power plant sites etc. (14, 100, 132).

1.8.2 In situ *bioremediation*

'On site' bioremediation can be separated in *ex situ* and *in situ* treatments, *in situ* meaning that the contaminated soil is remediated at its original place, whereas for on site *ex situ* treatments the soil is excavated but treated at the contaminated location by means of landfarming, composting, above ground bioreactors etc. (39). *In situ* treatments include biostimulation, bioventing, bioaugmentation, and natural attenuation (100):

Biostimulation: Biodegradation is enhanced by addition of oxygen, water and mineral nutrients (usually a combination of N, P and occasionally trace metals) (121). Oxygen may be added as H_2O_2.

Bioventing: Bioventing is the process of aerating subsurface soils to stimulate *in situ* bioremediation of less volatile compounds, while VOCs are simultaneously removed (129).

Bioaugmentation: This method involves the direct application of microorganisms originating from the remediation site, an off-site vendor or genetic engineering *in situ*. The microorganisms have been cultured and adapted. Their degrading ability can be enhanced for specific contaminants and site conditions (121).

Natural attenuation: Natural attenuation is the removal of contaminants by natural processes such as degradation, adsorption, and transformation. For natural attenuation to be a viable approach it is necessary to ensure by monitoring that the contaminants of concern are removed at reasonable rates (129).

The essential criteria that have to be met before *in situ* bioremediation can start are: the subsurface matrix must be permeable enough to allow perfusion with a solution of oxygen and nutrients, and contaminant degrading microorganisms must be present (151). If these criteria are met a thorough site investigation with regard to hydrogeology and contaminant regime has to be performed. This is on one hand based on laboratory tests to determine the nutrient requirements, optimal moisture content, pH and on the other hand on

site investigations to test the nutrient formulations with the subsurface properties. Toxicology assessment is required to evaluate the possibility of harmful biological effects of excessive nutrient application (100). System design, system operation and monitoring have to be planned according to the investigation results (151).

1.8.3 Monitoring the efficacy of bioremediation

During the bioremediation process it must be demonstrated that the contaminants are detoxified or preferably mineralised at the field site and finally that the clean-up criteria are reached (80). Monitoring ideally enables to establish complete mass balances and the verification of predictive, kinetic models and biodegradation processes. Monitoring usually includes measurements of chemical as well as microbiological parameters such as (77, 129)

- biomass indicators (*e.g.* total counts, PLFA and DNA quantification, plate counts)
- numbers of specific organisms (e.g *in situ* hybridisation, bioluminescent reporter bacteria, most probable number technique (MPN))
- physiological or genetic diversity (*e.g.* Biolog™, DGGE or TGGE, T-RFLP)
- activity (CO_2 / CH_4 production, oxidant consumption (O_2, NO_3^-, SO_4^{2-}), contaminant decrease, or metabolite production)

Biolog™ plates, which were applied in Chapters 2 and 3, was the only miniaturized physiological method commercially available at the beginning of this thesis and served as a starting point for all further improvements done in this work. Therefore they are presented in the following paragraph.

1.8.4 Assessment of physiological diversity with Biolog™ plates

A widely used tool to visualize functional changes in a soil community is the Biolog™ substrate utilization test. This test was originally developed for medical microbiology to identify single strains (22). It consists of a polystyrene 96 well microtiter plate. Each well contains a minimal growth medium, a tetrazolium dye, which changes colour when it is reduced by bacterial electron carriers, and a single substrate (22). According to the substrate degrading capabilities of a soil community inoculated on Biolog™ plates, a characteristic colour pattern is produced. This method has been widely applied in the last few years since it is commercially available and easy to apply. Applications included the study of microbial community changes in ground water (59), in the rhizosphere (31, 61, 69, 141) or phyllosphere of plants (78), in garden waste compost (87) and diesel oil contaminated

soil (28). A number of limitations associated with Biolog™ plates used for community analysis have been reported:

- Quantitative and representative recovery of microorganisms from environmental samples is required. A number of binding forces, including electrostatic and van der Waals forces, hydrogen bonding and physical entrapment (107) need to be overcome in order to reduce cell-soil associations and allow extractions of cells. Chemical (anionic detergents, ion-exchange resins) and physical (shaking, blending, sonication) dispersion treatments are often used, but even with exhaustive multi-stage extractions, large proportions of bacterial populations remain associated with soil particles (81).
- The Biolog™ plates have to be inoculated with a high cell density: $2\text{-}3 \cdot 10^8$ viable cells ml^{-1} are recommended by the manufacturer (Biolog Inc. Hayward, California). Below this density, there will be a lag time in colour development while the cells grow and achieve this concentration (71). Particularly for subsurface soil extracts researchers find less viable cells than required for immediate coloration and in that case Biolog™ becomes a culture based method. As mentioned above, less than 1 % of soil bacteria can be cultured to date (155).
- The importance of bacterial growth for color formation indicates that the responses of the community-level physiological profiling is a reflection of functional potential rather than *in situ* functional ability (62).
- The plate medium as well as the substrate selection was developed for medical applications, and nutrient concentrations are unsuitable for oligotrophic soil organisms.

Campbell *et al.* (31) composed their own substrate set, consisting of 30 carbon sources that were known components of plant root exudates. These substrates were applied to Biolog™ MT plates, which are purchased containing only the tetrazolium dye and the mineral medium. They discovered that the use of fewer carbon sources, which are more ecologically meaningful, were more efficient in discriminating between groups of samples from grassland sites with three different vegetation types than the standard GN plates. But there is a need for techniques allowing to characterize microbial communities without the usual reliance on extraction and subsequent selective culturing (96).

1.9 Objective and outline

Microorganisms are the main actors during bioremediation of petroleum hydrocarbon contaminated sites and care should be taken of them. This thesis aimed at investigating

microbial communities in soils that have been contaminated by VOCs and are undergoing a bioremediation treatment. The primary aim was to develop a rapid, easy, and cost-effective method to assess community changes without the main shortcomings of commercially available Biolog™ plates. Two different systems were modified in this work and adapted to the use with volatile hydrocarbons. They were applied with artificially contaminated soils to investigate increasing numbers of hydrocarbon degraders, assessment of community changes, hydrocarbon concentrations having toxic effects on soil communities, and optimal nutrient concentrations to achieve high degradation rates.

Chapter 2 discusses the development of biomass, activity, and community-level physiological profiles (CLPP), assessed before and after emplacement of a volatile organic contaminant source on the occasion of a field experiment in the vadose zone. Here, CLPPs were obtained by applying the classical Biolog™ method. This field experiment was carried out in the frame of the European project Groundwater risk assessment of contaminated sites (GRACOS). The biological parameters were related to site-specific characteristics and time dependent decontamination.

Chapter 3 describes developments towards a most-probable number (MPN) method for VOC degrading bacteria in artificially contaminated soils assessed on microtiter plates. A new tetrazolium dye was applied to decrease the number of bacteria required to induce a signal. The method is more sensitive than Biolog™. Applied materials were critically examined and conclusions for improvements in the following experiments were drawn.

Chapter 4 describes the application of a new micro-respiration system to investigate contaminant specific CLPPs with VOCs as substrates. Therefore whole soil samples were filled into 96-well microtiter plates. A pH indicator to detect CO_2 release was used to assess microbial activity. Toxicity responses on increasing VOC concentrations of a long-term contaminated subsoil and an artificially contaminated surface soil were compared.

Chapter 5 presents the micro-respiration system for assessing optimal nutrient requirements to enhance biodegradation of volatile petroleum hydrocarbon in a nutrient-deficient contaminated soil. The whole soil samples were nutrient- and substrate amended. Release of $^{14}CO_2$ due to degradation of a ^{14}C-labeled substrate was used as indicator of suitable nutrient composition. It is shown how the data may be fitted into a Monod equation to subsequently calculate optimal nutrient concentrations.

CHAPTER 2

Microbial community response to petroleum hydrocarbon contamination in the unsaturated zone at the experimental field site Værløse, Denmark

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2.1 Introduction

Contamination of soils and the underlying subsurface by petroleum spills is a widespread environmental problem. *In situ* bioremediation (14) for a cost-effective environmentally friendly clean-up and monitored natural attenuation (167) for risk management are two frequently applied strategies to cope with petroleum spills. Both rely on the potential of the autochthonous microbial communities to biodegrade petroleum hydrocarbons, which is supposed to be ubiquitous in oxic environments (128). The addition of hydrocarbon-degrading microbes is usually not more effective for hydrocarbon removal than stimulating the growth of the indigenous microorganisms, and indeed, inoculation is usually not practiced. A central question at polluted sites undergoing enhanced *in situ* bioremediation or natural attenuation is therefore the impact of petroleum contamination on the activity

and on the structure of the indigenous microbial communities in the subsurface. Previous work, *e.g.* (76, 126, 140, 143), focused on the bioremediation of petroleum-contaminated topsoils, covering microbiological, chemical and engineering aspects. The effects of petroleum pollution on terrestrial microbial communities have been studied at different experimental scales: in enrichment cultures (68), microcosm experiments (28, 130), soil columns (142), lysimeters (41, 60) and field plots or transects (50, 140, 173). Various methods such as community-level physiological profiles (CLPP) (45, 126, 172), phospholipid fatty acid (PLFA) profiling (28, 140), and nucleic acid-based methods (36, 50, 101, 165) have been used to study the composition of the microbial communities. Widmer and coworkers (166) applied three different methods (CLPP using Biolog™ GN plates, PLFA, and restriction fragment length polymorphism (RFLP) analysis) to evaluate the community structure in three different non-polluted agricultural soils. All three methods allowed distinguishing the soils based on fingerprints. After contamination of pristine soil with petroleum, an increase in microbial activity, but a decrease in diversity, is generally observed (28, 41, 50, 60, 68, 130, 142, 173). However, most studies with artificially polluted soils were performed in the laboratory, and significant effects on the activity and composition of the microbial communities in controls without contamination were also observed, as a result of the incubation process (26, 28). Thus the transferability of long-term laboratory studies to the field situation is often questionable. It can be concluded that the best way to exclude incubation bias is to work *in situ*.

The unsaturated or vadose zone is a fairly unknown compartment of the soil ecosystem (5). Before the 1970s a general agreement on a sterile or sparsely populated subsurface prevailed (34). Since then, studies characterizing the microbial communities of deeper vadose zones between topsoil and groundwater were primarily focused on the influence of the water potential on the abundance and activity of microbial organisms (see (5) for review). Consistently with lower carbon and nutrient fluxes, as compared to aquifers and topsoil layers, microbial activity and diversity is lower in the intermediate unsaturated zone. At a pristine site in Oklahoma with an unsaturated zone of 3 m thickness (20), biomass and substrate induced-activity of bacteria and protozoa were constant in summer and winter, but varied sharply with depth, with a minimum in the deep unsaturated zone above the capillary fringe. In a pristine unsaturated zone of 26 m thickness in the US Midwest, the lowest biomass and smaller biodegradation rates of glucose and phenol were found in unsaturated tills as compared with both topsoil and the deeper saturated zone (99). Only a few field studies (19, 173) report on microbial populations in vadose zones at petroleum spill sites. In most studies the petroleum is floating on the groundwater and investigations were limited to the deep vadose and the saturated zone. The significance of the vadose zone for petroleum biodegradation remains unclear and better characterization is needed at contaminated sites. Pasteris *et al*. (123) performed a lysimeter experiment simulating

an unsaturated zone above a groundwater table using pristine alluvial sand as a porous medium. The CO_2 concentration in the soil increased the day after addition of petroleum hydrocarbons and was linked to aerobic biodegradation of hydrocarbons and growth of microbial biomass. Hence the microbial community in the sand adapted within a short time to petroleum, and biodegradation started rapidly. However, the unsaturated alluvial sand had been disturbed physically during filling of the lysimeter, potentially activating the microbial community before contamination.

The main aim of this study was to monitor the activity and structure of an indigenous microbial community in a 3.3 m deep natural sandy unsaturated soil before and after artificial contamination with petroleum hydrocarbons. Biolog™ EcoPlates results were used together with redundancy analysis (RDA) in order to test the relationship between functional diversity (using the term as defined in (125)) and site-specific variables. Detailed results on hydrocarbon migration were measured by P. Kjeldsen *et al.* (Technical University of Denmark, unpublished data).

2.2 Materials and methods

2.2.1 Experimental site and contamination

The field experiment was conducted from July 2001 to July 2002 at the site of the European project GRACOS at Airbase Værløse in Denmark, which is a location without prior contamination by petroleum products. At this site, a sandy dark brown topsoil of 0.3-0.5 m thickness is overlaying 2.0-3.3 m of homogeneous glacial melt-water sand, which in turn overlays 0.5-1.0 m of moraine sand/gravel, having visible white lime. An unconfined aquifer was found in July 2001 at a depth of 3.3 m. The water table was shown to rise between 1.2 and 1.6 m during wet winters. The site was covered with grass and had not been fertilized over the course of at least 50 years.

An artificial petroleum hydrocarbon mixture of 13 typical kerosene hydrocarbons (Table 2.1) was composed from pure products, to which the chlorofluorocarbon 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) was added as a conservative volatile tracer. To form a source of hydrocarbons, 10.2 liters of this mixture were mixed with about 200 liters of sand from the site and buried at a depth of 0.8 to 1.3 m below the surface in a round hole of 0.70 m diameter. After filling the rest of the hole with the original soil material, a lid of 1.24 m diameter was installed 0.3 m above ground in order to prevent direct rainwater infiltration into the buried source. The migration of hydrocarbon vapors and the composition of the soil gas were monitored with high spatial and temporal resolution for the subsequent 12 months after contamination. Stainless steel capillaries (1/16“) were installed

in the experimental area before kerosene source burial (Figure 2.1). The compounds in the pore-gas were sampled on Tenax® packed sorbent tubes and analyzed by automated thermal desorption coupled with a gas chromatograph equipped with flame ionization and electron capture detection (ATD-GC-FID and -ECD). Soil gas and groundwater were sampled along a main sampling axis within 20 m from the source center. The groundwater flow direction, determined before the experiment, defined the main sampling axis (Figure 2.1). Hydrocarbon data measured along this main axis and along three secondary axes (90°, 180° and 270° to the main axis) showed that vapor migration was generally isotropic. The soil temperature was measured with fixed installed thermometers to a depth of 2.5 m.

Table 2.1: Composition of the oil phase after site installation.

Compound	Amount (wt%)	Compound	Amount (wt%)
Benzene	1.02	*n*-Dodecane	9.50
Toluene	2.93	3-Methylpentane	7.45
m-Xylene	4.57	Isooctane	15.36
1,2,4-Trimethylbenzene	10.99	Cyclopentane	1.59
n-Hexane	7.26	Methylcyclopentan	5.79
n-Octane	7.16	Methylcyclohexane	10.23
n-Decane	15.99	CFC-113	0.16

At sampling distances of 0.7 m (cores A, D and G), 2 m (B, E, H) and 10 m (C, F, I) from the source center (for the spatial arrangement of coring sites see Figure 2.1), three soil cores were sampled on three different days. Soil cores A-C were taken on the 24th day before contamination, soil cores D-F and cores G-I on the 23rd and 80th day after contamination of the site, respectively.

The soil cores D, E, G, and H were taken within the vapor plume. Microbial populations in these cores had been more or less steadily exposed to hydrocarbon vapors for about 20 days (cores D+E) and 77 days (cores G+H). At 10 m from the source, most hydrocarbons were at all times below the detection limits, except *n*-hexane, isooctane, cyclopentane, methylcyclopentane, methylcyclohexane and 3-methylpentane which were detected in traces between day 13 and 17. Therefore, the microbial populations in samples from the soil cores F and I can be viewed as uninfluenced by petroleum hydrocarbons, in addition to those of soil cores A-C taken prior to contamination. A maximum petroleum vapor mass in the soil pore air of 450 g was reached after 17 days and then decreased to 2 g on day 350. The vapor concentrations of individual components generally decreased three orders of magnitude within 2-8 m lateral distance from the buried source. Only after 113 days did the most volatile compounds (cyclopentane, 3-methylpentane, methylcyclo-

pentane) disappear completely from the vadose zone at 2 m distance from the source center, and some compounds persisted at the site for more than one year.

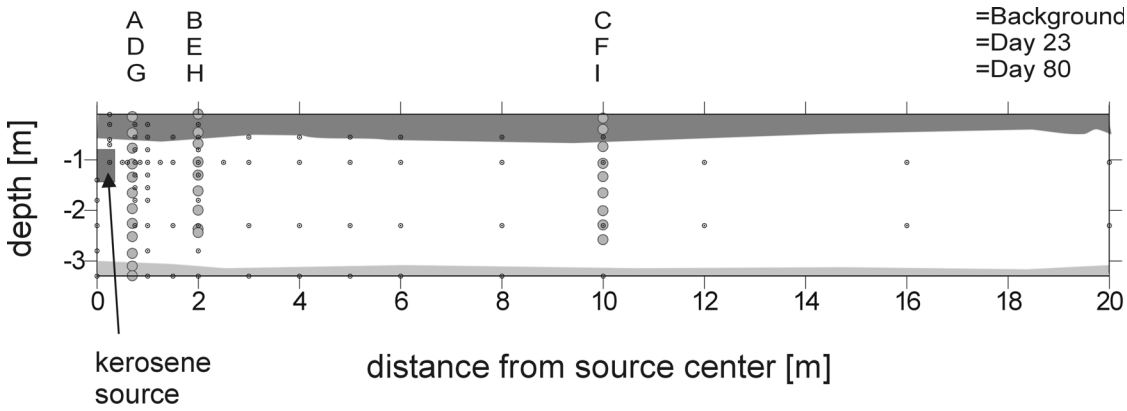

Figure 2.1: **Cross-section of field site, with location of kerosene source (dark grey square), soil gas sampling ports along the main sampling axis (black circles), and location of soil cores drilled along an axis of 120-140° to the main sampling axis (grey dots). Soil cores A-C were taken on the 24th day before contamination, soil cores D-F and cores G-I on the 23rd and 80th day after contamination of the site, respectively, at distances of 0.7 (A, D and G), 2 (B, E, H) and 10 m (C, F, I) from the source center. For depths of soil samples see Table 2.2.**

2.2.2 Soil sampling

In order to avoid any disturbance of the soil by the installed gas or water sampling devices, soil samples were taken only at locations outside the buried petroleum and along an axis at an angle of 120-140° to the main sampling axis. Soil cores were taken using a stainless steel hollow-stem soil corer of 2.8 cm diameter (LB system, Geoprobe, Salina, Kansas). The corer was driven into the ground by a motor hammer until the moraine gravel made further lowering impossible. Undisturbed samples were obtained in PVC liners of 60 cm length. The top 10-15 cm in each liner were discarded to exclude topsoil, which had fallen into the hole. Soil samples were transferred on site into sterile PE tubes and stored at soil temperature (15±3°C) for a maximum of 3 days before they were homogenized, sieved (<2 mm), and further analyzed. After coring, each remaining hole was filled with clean sand and bentonite.

2.2.3 Carbon dioxide and oxygen measurements

Soil gas for the measurement of oxygen and carbon dioxide was sampled at 107 gas sampling tubes represented as black circles in Figure 2.1. These probes were purged of dead air before samples of 3 ml were withdrawn with a syringe.

Table 2.2: Selected properties of the soil samples.

Core A, background								Core D, day 23								Core G, day 80							
Label	Depth [m]	pH	TOC [g/kg]	N tot [g/kg]	P tot [g/kg]	Wat Cont[a]	HCarb[b] [g/m^3]	Label	Depth [m]	pH	TOC [g/kg]	N tot [g/kg]	P tot [g/kg]	Wat Cont[a]	HCarb[b] [g/m^3]	Label	Depth [m]	pH	TOC [g/kg]	N tot [g/kg]	P tot [g/kg]	Wat Cont[a]	HCarb[b] [g/m^3]
A1	-0.23	5.3	9.5	0.822	0.56	0.113	0.00	D1	-0.24	5.0	4.0	0.281	0.73	0.071	0.65	G1	-0.30	5.7	8.3	0.664	0.61	0.162	2.16
A2	-0.55	6.2	2.6	0.166	0.62	0.132	0.00	D2	-0.57	6.0	3.5	0.214	0.71	0.108	13.44	G2	-0.55	6.1	2.2	0.189	0.51	0.188	5.74
A3	-0.85	6.4	1.0	0.050	0.24	0.074	0.00	D3	-0.88	6.5	1.8	0.089	0.27	0.068	20.81	G3	-0.85	6.5	0.8	0.046	0.27	0.110	11.33
A4	-1.16	6.3	1.9	0.093	0.30	0.057	0.00	D4	-1.18	6.6	0.6	0.029	0.21	0.043	62.24	G4	-1.20	6.5	0.4	0.042	0.23	0.112	19.56
A5	-1.42	6.6	1.1	0.045	0.26	0.078	0.00	D5	-1.39	6.6	0.6	b. d. l.	0.24	0.079	99.73	G5	-1.45	6.7	0.3	b. d. l.	0.15	0.017	16.79
A6	-1.73	6.8	0.6	0.029	0.20	0.104	0.00	D6	-1.75	6.7	0.6	0.013	0.21	0.082	36.65	G6	-1.75	6.8	0.4	0.030	0.21	0.115	15.39
A7	-2.04	6.7	0.7	b. d. l.	0.16	0.069	0.00	D7	-1.97	6.5	5.1	0.375	0.49	0.078	47.27	G7	-2.00	6.7	0.3	0.016	0.18	0.089	11.62
A8	-2.33	6.7	b. d. l.	b. d. l.	0.15	0.107	0.00	D8	-2.27	6.7	0.4	b. d. l.	0.19	0.066	42.65	G8	-2.35	6.7	0.3	0.013	0.18	0.101	6.33
A9	-2.59	6.8	0.6	b. d. l.	0.14	0.076	0.00	D9	-2.63	7.0	0.7	0.020	0.18	0.078	-	G9	-2.60	6.8	0.3	0.025	0.15	0.079	-
A10	-2.92	6.9	0.6	b. d. l.	0.22	0.190	0.00	D10	-2.90	6.8	0.7	0.012	0.16	0.164	-	G10	-2.90	7.1	0.5	0.018	0.22	0.133	-
A11	-3.17	7.0	1.1	0.041	0.20	0.096	0.00									G11	-3.20	8.6	0.4	0.033	0.29	0.054	-
A12	-3.36	8.8	1.3	0.046	0.34	0.228	0.00																

-: not measured

[a]: Wat Cont is water content (v/v),

[b]: HCarb means hydrocarbon (kerosene) content in the soil gas

b. d. l.: below detection limit

The gas samples were stored in evacuated blood collection tubes (Venoject tubes, Terumo, Leuven, Belgium) and analyzed using a portable Chrompack Micro GC (Middelburg, The Netherlands). The method has been previously described in (35). Since the sampling days for CO_2 and O_2 did not correspond to the sampling days of the soil cores, adjustment was allowed for gas data by linearly interpolating between consecutive dates. Linear interpolation was considered accurate enough since the delay between gas sampling days and the core drilling never exceeded more than 3 % of the total duration of the experiment. For the depths of the samples A10, A11, A12, D10 and G10 no CO_2 values were available. Average values of the corresponding cores were used for statistical analysis instead. Contour plots were drawn with Surfer 7.00 (Golden Software Inc., Colorado, USA) using Kriging for interpolation.

2.2.4 General soil characteristics

Soil pH (free acidity) was measured in H_2O (soil to water ratio 1:2.5 w/w) after shaking for 1 hour. All soil samples were ground, mineralized by the method of Kjeldahl, and analyzed colorimetrically for total nitrogen (N_{tot}) and phosphorus (P_{tot}) on a Technicon Autoanalyzer II (Dublin, Ireland). Total soil carbon was combusted at 1000°C to CO_2 which was analyzed on a CASUMAT analyzer (Wöstmann, Bochum, Germany). The CO_2 release from soil boiled in 33 % phosphoric acid was measured in order to get the total inorganic carbon content and the total organic carbon content was calculated by subtracting the inorganic carbon content from the total carbon content. The temperature profiles in the soil were measured on selected days at 4 p.m. with buried thermometers. Missing values were interpolated from the closest measurements. Water content was measured by weight loss due to evaporation after 24 hours at 105°C.

2.2.5 Direct counts of soil bacteria

For enumeration of total bacteria, the <2 mm sieved fraction of 0.3 g of each soil sample was suspended in 4 % paraformaldehyde, washed with phosphate buffered saline (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2) and stored in 96 % EtOH at –20°C (173). The suspensions were then diluted between two and ten fold (depending on cell density) in pyrophosphate buffer (0.1 % $Na_4P_2O_7 \times 10\ H_2O$), and ultrasonicated to disrupt biofilms and permeabilize cell membranes. Three times 20 µl of the dispersed suspension were spotted on gelatin-coated slides dried on heating plates at 70°C and finally dehydrated sequentially in 50 %, 80 % and 96 % ethanol for 3 min each. The dry samples were stained for 10 min with an aqueous mixture of 4',6'-diamidino-2-phenylindole hydrochloride (DAPI, 5 µg ml^{-1}) and acridine orange (AO, 0.001 %), dried again, covered with a drop of citifluor AF1 (Citifluor Ltd., London, UK) and a cover slip and stored at –20°C.

Bacteria were counted with a microscope (Olympus BX-60, Olympus Optical Co. Ltd., Tokyo, Japan) equipped for epifluorescence. Staining with a DAPI/AO mixture allowed easy distinction between orange-stained soil particles and blue-stained bacteria. Cell counts are average cell numbers of 16 equally distributed fields on a microscope slide. Averages and standard deviations presented in Figure 2.3 were calculated from three of these measurements based on 16 fields.

2.2.6 Protein quantification in soil samples after Bradford

Protein was quantified in order to test the correlation between total cell numbers and microbial biomass. To prepare 1 l of Bradford Reagent, 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml ethanol (96 %). Deionized, filtered H_2O and 100 ml H_3PO_4 (85 %) were added to give a volume of 1000 ml. Protein standards in the range of 0.5-0.005 mg/ml were prepared from a stock solution of 1 mg bovine serum albumin (BSA) per ml. Sieving soil samples (<2 mm) allowed to separate roots and worms from the soil before protein analysis. One ml of 1 M NaOH was added to 1.5 g sample soil and cooked for 10 min at 95°C (173). The soil particles were subsequently settled by centrifugation (5 min at 4000 rpm). After chilling, 0.5 ml of the supernatant was neutralized by addition of 1 M HCl (sterile, filtered) in a new tube. The OD_{590} was measured after incubation for 5 min with Bradford reagent. Particles and dissolved material was accounted for by a blank for each sample. Since the pH was lowered to 1 by addition of the Bradford reagent to the sample, some soil material was dissolved. To have the same conditions in the blank and in the sample, a solution prepared identically to Bradford reagent without Coomassie Brilliant Blue was added.

2.2.7 Characterization of community-level physiological profiles

Microorganisms for physiological profiles were extracted by shaking 30 g soil for 30 minutes in 75 ml sterile deionized water at 180 rpm on a rotary shaker. Deionized water was chosen, since, according to the DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory of colloid stability, low ion concentration favors detachments of particles from surfaces (134). The samples were allowed to settle for 10 min. Ten ml suspensions were put on ice until used. To estimate inoculated cell numbers and extraction efficiency, 100 µl of the suspension was stained by 20 µl of a DAPI/AO mixture described above, filtered and dried in ethanol on a polycarbonate filter. Extracted and inoculated cell numbers were determined by fluorescence microscopy. The extraction efficiency of 18-80 %, expressed as percentage of total count cell numbers, strongly depended inversely on the organic matter content. CLPP were obtained from all samples of cores A, D, and G, and from a few selected samples of the remaining cores taken at the depth of the buried source.

Biolog™ EcoPlates (Biolog Inc., Hayward CA, USA) (88) are commercially manufactured microtiter plates loaded with mineral medium, a tetrazolium dye and 31 different carbon sources in triplicates. Community-level physiological profiles (CLPP) are based on reduction of the dye linked to carbon source utilization in the wells of those plates (168). None of the carbon sources is a fuel compound or expected to occur significantly in hydrocarbon-polluted soils due to contamination. By means of a 8 channel pipette, one plate per sample depth was inoculated with 125 µl per well of the 1:100 (topsoil) or 1:19 diluted extract. Plate incubation occurred at steady temperature of 21° C in the dark, absorption was automatically measured at 595 nm every 12 hours with a plate reader (Dynex Technologies Inc. MRX II, Chantilly, USA). Values of the respective blanks were subtracted and negative values that occasionally resulted were set to zero. Average well color development (AWCD) was calculated for each well but not used for further data transformation for reasons described in the results section. Values after 72 h incubation and absorbance areas under curves to 120 h were used for further analysis. The areas were obtained by calculating a 'Riemann's sum' as also suggested in (110). The substrate richness, S, is the total number of utilized C-sources out of 31 and utilization being ascribed to absorbance values of samples minus absorbance of blanks exceeding 0.25 after 72 h. A coefficient of variation (CV) was introduced in order to gain information on the reliability of the mean values of the three substrate replicates on one plate. The percentages of all 31 plate substrates with a $CV=\sigma\ \mu^{-1}$ (σ being the standard deviation, μ the mean value of the three replicates) smaller than 100 % are listed in Table 2.3.

2.2.8 Statistical treatment of data

The whole data set of 33 objects/samples was subdivided in five files according to the variables: 'Biolog 72h' (absorption after 72 hours: 31 quantitative variables), 'Biolog area 120h' (areas under absorbance curves after 120 hours: 31 quantitative variables), 'Biology data' (CO_2 concentration, log of cell numbers, protein quantification: 3 quantitative variables), 'Soil parameters' (depth, pH, temperature, N_{tot}, P_{tot}, total carbon, TOC and volumetric water content: 8 quantitative variables), and 'Time' (days after contamination, given as three binary variables). In order to quantify and test effects of various sets of explanatory variables on the Biolog and Biology data variation, Redundancy Analysis (RDA) was applied using the CANOCO 3.12 software (148, 149). RDA is a canonical method that allows simultaneous analysis of two or more data tables. Like Principal Component Analysis (PCA), RDA is used to calculate ordination of samples, with the difference that in RDA the explanatory variables intervene in the ordination of the response variable matrix. The ordination vectors are forced to be maximally related to linear combinations of the explanatory variables (103) and the canonical model can be tested. Variables of the data matrix have been centered by CANOCO, those of the explanatory

variables were standardized. A correlation matrix was used to map the various types of variables in the data matrix. During the analysis the most discriminating variables were selected by the 'forward selection' procedure of the program. Statistical tests were run using the Monte Carlo permutation procedure of CANOCO (29). The same statistical package was used for preliminary PCA.

2.3 Results

2.3.1 Soil characteristics

Results from chemical soil analysis are presented in Table 2.2. The total organic carbon content (TOC) declined from 4-9 g kg^{-1} in topsoil to less than 1 g kg^{-1} in the vadose zone at 80-130 cm depth where the contamination was buried. However, in selected samples such as D7 at 1.97 m depth visible organic matter lenses led to a high TOC content. Total nitrogen and phosphorus were distributed similarly to TOC. Volumetric soil water content was influenced by recharge in the first meter, but remained stable at a depth of 2.50 m. The maximum water content was found around 2.9 m and 3.3 m depth in cores with low recharge (A and D). The soil pH showed a distinct vertical gradient with values ranging from 5 in the topsoil to nearly 7 in the sandy unsaturated zone. However, pH values higher than 8.5 caused by carbonate minerals in the moraine gravel were found at 3.36 m depth in core A and at 3.2 m depth in core G.

2.3.2 Microbial activity

Carbon dioxide (Figure 2.2) was chosen as the factor indicating microbial activity in the vadose zone. Background CO_2 concentrations in the soil gas were heterogeneously distributed in the depth profile, but were lower than 0.9 % everywhere (Figure 2.2). A maximum was found in the topsoil at 4 m horizontal distance due to mineralization of natural soil organic matter. On day 22 after contamination, a maximum of 1.24 % was found below the buried source, at 3.3 m depth just above the groundwater table, suggesting an increased mineralization activity in the capillary fringe below the source. On day 71 highest CO_2 concentrations were found at 1 m distance between 0.3 and 0.8 m depth as well as at 3.3 m depth. The maximum values of 2.12 % were found at day 87 at the same location as on day 22. There was a distinct lateral gradient of CO_2 more than 10 m radially away from the source. The measurements of CO_2 at 20 m horizontal distance showed that there was a seasonal variation in the CO_2 concentrations. The O_2 consumption (data not shown) was small and correlated to CO_2 production indicating that mineralization was essentially linked to aerobic respiration. The measurements ubiquitously showed more than 17 % O_2 in the soil air.

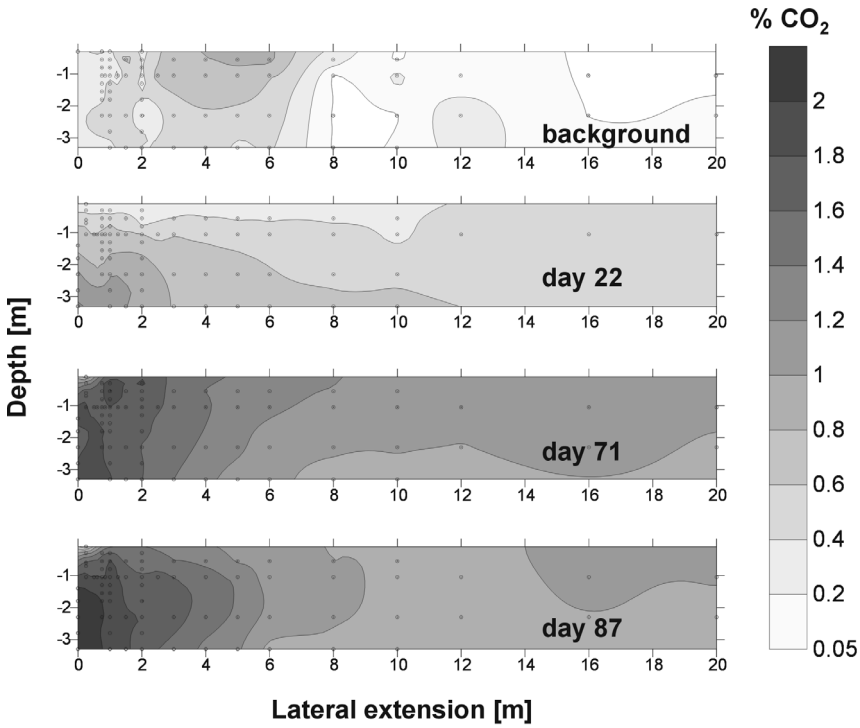

Figure 2.2: **Contour plots of CO_2 concentration (v/v) in soil gas measured 7 days before (background), 22, 71, and 87 days after the burial of the artificial fuel mixture.**

2.3.3 Distribution of microbial cells

The total number of cells counted before contamination decreased from $7 \cdot 10^8$ -$3 \cdot 10^9$ cells g^{-1} in the topsoil to 10^7 -$4 \cdot 10^7$ cells g^{-1} in the sandy vadose zone between 140 and 280 cm depth (Figure 2.3). Increased cell numbers were found in samples taken from the capillary fringe (samples A11 and G11). In the saturated zone the numbers decreased again (sample A12, Figure 2.3). Two samples of the vadose zone of core D contained unexpectedly high numbers of bacteria: At 2 m depth the corer was drilled across a local organic matter lens easily visible by eye. Organic matter, N, and P analyses confirmed the observation (Table 2.2). The reason for the occurrence of high bacterial numbers at the depth of 2.6 m was less evident, since no visible structural change accompanied the observed higher contents of organic matter, P and N. Cell numbers were best correlated with the N_{tot} (r^2 for all cores >0.9, $p<0.001$) and organic matter content. Protein and cell counts were generally well correlated with the exception of some surface samples where the protein content was underestimated.

2.3.4 Community-level physiological profiles

Within a core the AWCDs were very heterogeneous and sometimes not exceeding a mean absorption of 0.4. To compare plates at such low AWCDs was not reasonable in this experiment since surface samples had already reached this value after a few hours, although bacteria had not started to mineralize all of the degradable substrates by that time. Since the cell numbers in the vadose zone were very low it seemed to be unreasonable to dilute extracts of other samples to the cell density of those poor ones, which would have led to losses of bacterial strains. Neither did we divide absorbance values by the AWCD since the activity of bacteria in surface soil and deeper vadose zone is very different, and we would have lost information as described in (83) and (125).

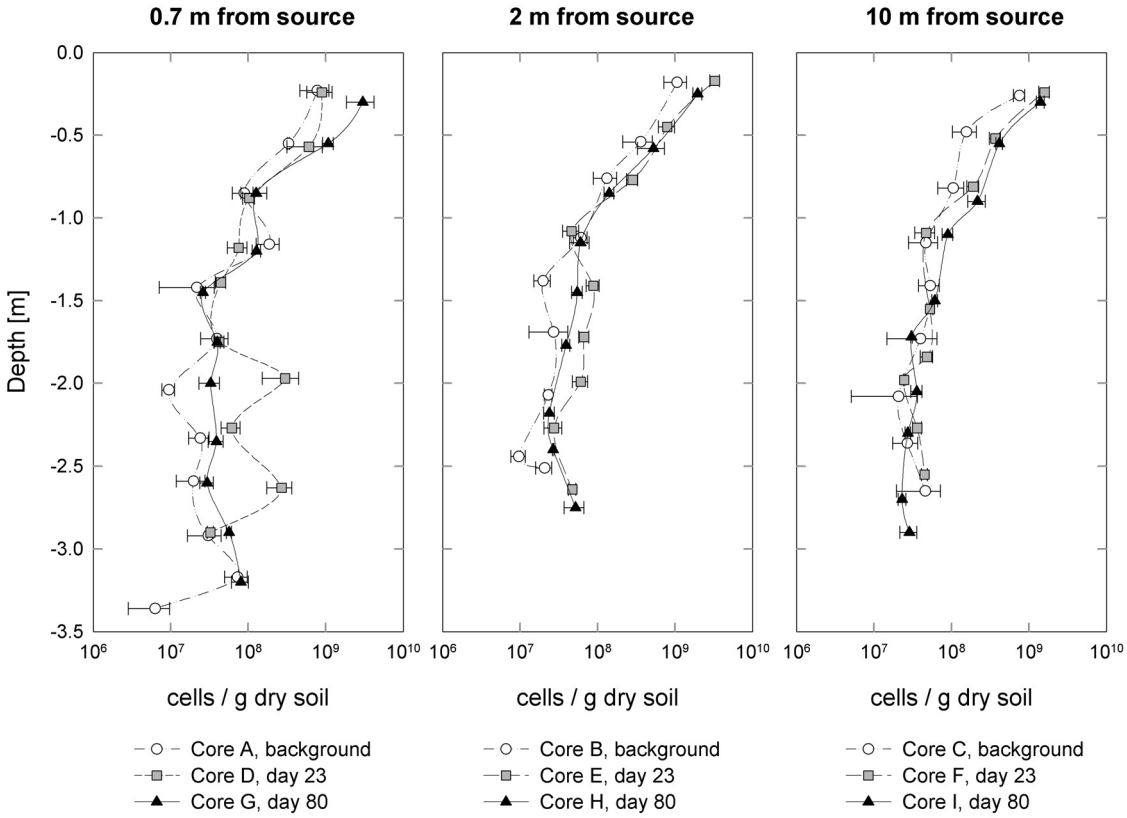

Figure 2.3: **Depth profiles of total microbial cell numbers measured 7 days before (background, A), 23 (D), and 87 days (G) after the burial of the artificial fuel mixture determined as direct microscopic counts by fluorescent microscopy. Error bars represent standard deviation.**

PCA profiles of the areas under the curve up to 120 hours were compared with those using data up to 72 h and 96 h and no obvious differences were detected, indicating that an incubation time of 72 hours was long enough to level out differences in inoculum densities.

The substrate richness (S) at 72 h is indicated in Table 2.3. All three cores show a similar pattern of S and coefficient of variation (CV). Generally, S and CV tended to be highest in samples with high organic matter content. Unexpectedly high values were, however, found in samples A11 and G11 from the capillary fringe, indicating high functional diversity. The low cell number in some of the deeper samples led to only few degraded substrates and to considerable variations between replicates.

Table 2.3: Substrate richness S and repeatability of the substrate use (CV) of samples analyzed on Biolog^{TM} EcoPlates.

Core A, background				Core D, day 23				Core G, day 80			
Sample ID	Depth [m]	Substrate richness (S)	Sum of CV<1*	Sample ID	Depth [m]	Substrate richness (S)	Sum of CV<1*	Sample ID	Depth [m]	Substrate richness (S)	Sum of CV<1*
A1	-0.23	18	65	D1	-0.24	18	58	G1	-0.30	18	68
A2	-0.55	18	81	D2	-0.57	22	81	G2	-0.55	20	61
A3	-0.85	19	56	D3	-0.88	17	52	G3	-0.85	17	71
A4	-1.16	22	68	D4	-1.18	16	55	G4	-1.20	14	36
A5	-1.42	19	55	D5	-1.39	13	49	G5	-1.45	16	61
A6	-1.73	6	10	D6	-1.75	15	45	G6	-1.75	3	3
A7	-2.04	21	74	D7	-1.97	22	71	G7	-2.00	17	42
A8	-2.33	22	58	D8	-2.27	10	32	G8	-2.35	13	36
A9	-2.59	20	52	D9	-2.63	17	52	G9	-2.60	16	56
A10	-2.92	20	23	D10	-2.90	17	36	G10	-2.90	12	13
A11	-3.17	28	100					G11	-3.20	24	68
A12	-3.36	16	58								

*Percentage of all substrates having a coefficient of variation smaller than 1

2.3.5 RDA ordination

The correlation structure between the variables 'Biology data', 'Soil parameters' and 'Time' is summarized in Figure 2.4, whereas Figure 2.5 shows the correlation structure between 'Biolog area 120h', 'Soil parameters', 'Time' and 'Biology data'. Hydrocarbon concentrations in soil gas were not used since the variance inflation factors in the canonical regression model were very high and the 13 variables (petroleum hydrocarbons) had to be reduced to two. Instead, the time can be used as an indirect measure of the exposure by hydrocarbon pollution. The variance in the 'Biology data' (Figure 2.4a), consisting of cell counts, protein quantification and CO_2 measurements, could be explained to 46 % by the 'Soil parameters', and 'Time' explained another 39.8 % of variance, 5.7 % of those values being shared. This result is significant overall ($p<0.001$). Forward selection was used in order to reduce the number of variables of 'Soil parameters' to approximately the same as in 'Time'. Variables explaining the largest statistically significant amount of variation were P_{tot} ($p<0.001$), N_{tot} ($p<0.001$), temperature ($p<0.005$), and pH ($p<0.046$).

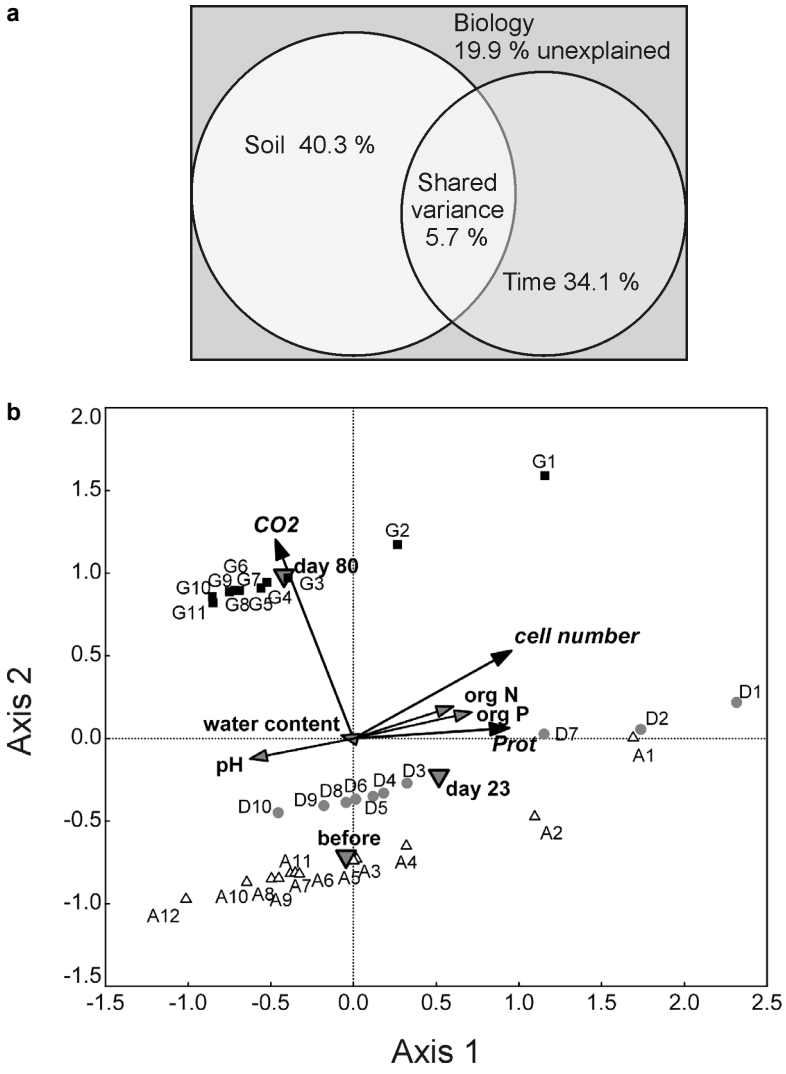

Figure 2.4: a) Variance decomposition of the Redundancy Analysis (RDA) of 'Biology data'. The percentage of variance explained by either the soil, or the contamination (expressed as time) are given.
b) Triplot diagram of the Redundancy Analysis (RDA) of 'Biology data'. Descriptors (arrows) are the number of cells per gram dry soil (cell number), μg protein g^{-1} dry soil (Prot) and the carbon dioxide emission (CO_2). Total nitrogen (N_{tot}), volumetric water content (water content), phosphorus content (P_{tot}) and pH (pH) are used as quantitative explanatory variables (arrows). Time 0 (before), day 23 and day 80 (after) are used as qualitative explanatory variables (centroids). Axis 1 (45.1 %, λ:0.451, p<0.001) and 2 (33.3 %, λ: 0.33, p<0.001) are represented. Total of explained variance in the correlative model is 80.1 %. Samples are labeled according to the time of contamination (A: before, D: 23 days after , G: 80 days after) and numbered according to increasing depth (for details see Table 2.2).

With these variables, 51.2 % of variance was explained, but we eliminated 'temperature' since some missing values were calculated. The next most significant variable, 'water

content', was instead included in the further RDA. Depth, TOC, total carbon (TC) were all highly correlated with the above-mentioned variables. In the triplots (Figure 2.4b and Figure 2.5b), quantitative variables are represented by arrows, qualitative explanatory variables (centroids) are indicated by their label. Black and grey arrows indicate response and explanatory variables, respectively. The angle between two arrows and between an arrow and an axis, or between an arrow and inter-connecting line between the origin and an object-point are proportional to the correlation. Distance between object-points and between centroid-points, or between each other, are interpreted respectively as a mutual similarity or contribution. The projection of a centroid-point onto an arrow is proportional to its contribution. High correlations between cell number, N_{tot}, P_{tot} and protein measurements and the first RDA axis are observed. The pH is negatively correlated with those variables. CO_2 is correlated with axis 2. It is remarkable that the arrow 'CO_2' is perpendicular to the arrow 'cell numbers', meaning that they are independent. Cores are clearly separated by CO_2, and, indeed, the centroid for day 80 is opposed to day 23 and the one representing the background. The samples of each core are arranged according to depth, selected soil parameters and biomass related variables.

In the analysis of 'Biolog area 120h' (Figure 2.5) the number of soil variables was reduced by forward selection for the same reason as described above. Again four variables were retained for the RDA: P_{tot} ($p<0.014$), depth ($p<0.036$), volumetric water content ($p<0.047$), and N_{tot} ($p<0.26$). The total explained variance in this canonical model is 42.7 %, 'Time' explains 9.45 %, 'Soil parameters' 27.05 %, 'Biology' 15.6 %. Shared variance occurred only between 'Soil parameters' and 'Biology' and was 9.4 % of the previously mentioned values. This result is overall significant ($p<0.007$). Descriptors which are well correlated with the two first RDA axes were either polymers or carboxylic acids, according to the grouping in (62). 'Biolog area 120h' data did not well separate the cores but arranged samples in the following ways: in the bottom right quadrant are samples with high organic matter, which proved to be positively correlated with the selected soil variables N_{tot} and P_{tot}, in the bottom left quadrant are samples, which were exposed to high petroleum concentrations and which is in accordance with the CO_2 variable (see Table 2.2), in the top left quadrant are deep samples with a high water content, and in the first quadrant are less contaminated samples with small cell numbers. A11 and G11 have rather high cell numbers, have a high pH and are situated between two soil layers with high volumetric water contents.

Variance in 'Biology data' in samples of all soil cores (A-I) at the source depth was to 98 % explainable by 'Soil parameters' when all variables were included. By reducing the 'Soil parameters' by forward selection but including 'Time' for explanation, 96.8 % of the

variance was explained with a significant overall model and one significant axis (data not shown).

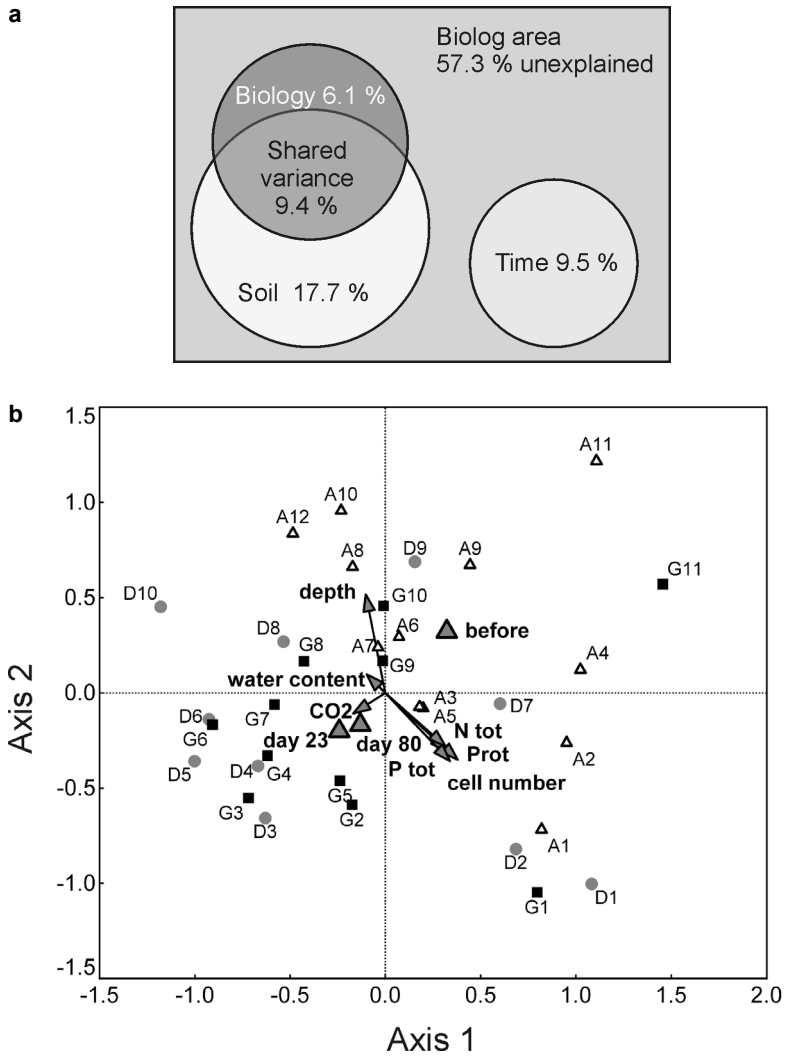

Figure 2.5: a) Variance decomposition of the RDA on 'Biolog area 120h' data. 'Soil parameter', 'Time' and 'Biology data' explain together 42.7 % of the total variance. b) Biplot diagram of the Redundancy analysis (RDA) of 'Biolog area 120h' data. Descriptors are the average areas under the absorption curves of the Biolog^{TM} plates after 120 h (not shown). Total phosphorous (P_{tot}), volumetric water content (water content), total nitrogen (N_{tot}), depth, protein quantification (Prot), log of cell numbers (cell number) and CO_2 emission (CO_2) are used as quantitative explanatory variables (arrows). Time 0 (before), day 23 and day 80 are used as qualitative explanatory variables (centroids). Axis 1 (23.3 %, λ:0.233, p<0.07) and 2 (10.3 %, λ:0.103, p > 0.1) are represented. Samples are labeled according to the time of contamination (A: before, D: 23 days after, G: 80 days after) and numbered according to increasing depth (for details see Table 2.2).

2.4 Discussion

2.4.1 Microbial activity and abundance vs. nutrient limitation

Effect of measurable CO_2 production became obvious only 16 days after contamination of our experimental field site. This was later than expected since the delay of CO_2 production in the lysimeter experiment described in (123) lasted only one day. The production of CO_2 was spatially closely related to the extension of the hydrocarbon vapor plume. However, although CO_2 concentration clearly increased at 0.7 m from the source (Figure 2.2), the bacterial cell numbers increased only insignificantly (Figure 2.3). This finding was unexpected since Ainsworth *et al*. (5) reported on 10^4-10^7 cells per gram of uncontaminated, dry soil and orders of magnitude higher cell numbers when contaminants were present at relatively high but not toxic concentrations. Zarda and co-workers (173) drilled cores through the unsaturated zone above a xylene plume. They found elevated numbers of bacteria and protists, especially in the xylene-affected zone just above the groundwater table and in the saturated zone. Pasteris *et al.* (123) observed increasing cell numbers in a lysimeter study after contamination by a similar petroleum mixture as the one used here, using the same counting method. Since in our study neither a significant increase in cell numbers nor a correlation between CO_2 production and cell numbers could be detected, it is likely that growth was limited by soil parameters such as nutrient concentrations and that degradation was essentially due to the metabolism of non-growing cells. Zhou and Crawford (175) showed that biodegradation of gasoline vapors in soil depended on the C:N ratio. The authors suggested that lower C:N ratios favored microbial growth. A good C:N ratio for their soils was 50:1 or lower. The natural ratio of TOC:N in the unsaturated zone of the Værløse soils is between 20:1 and 50:1. Taking into account the hydrocarbon contamination, which exceeds 1780 ppm described in (175), the ratio becomes higher than 50:1 and is thus outside the reported optimal range. Batch experiments carried out with Værløse soil showed increasing degradation when ammonium vapor was added (N. Dakhel and P. Höhener, personal communication), which confirms the assumption of N-limitation. This finding is in agreement with the better correlation of cell numbers with N_{tot} than with any other soil parameter ($r^2 > 0.9$) and is concordant with results of (8), who found N limitation for toluene degradation in subsurface soils.

2.4.2 Parameters influencing community size, activity and structure

The forward selection for the RDA model assessed the following order from the most to the least explicative variable of the 'Biology data': $P_{tot} > N_{tot} >$ temperature > pH > water content > TOC > C_{tot} > depth. PLFA data ordination in a study from (15) about sheep grazing on grassland shows that the microbial biomass variation among soils as deter-

mined by PLFA quantification is mostly correlated to differences in organic matter content and soil pH. Cell numbers in our study correlated negatively with pH. N_{tot} and TOC were highly correlated so that only the former was used for RDA. Neither CO_2 nor hydrocarbons were detected in topsoil since the gases diffused out to the atmosphere. Hence topsoil samples in Figure 2.4b) are apart from the CO_2 axis. The uncontaminated core A is more dispersed along the cell number-pH axis than contaminated samples. Samples A11 and G11, which are rich in nitrogen and bacteria and have a lower water content than their neighboring samples, are not clearly grouped apart of the other data points in Figure 2.4b. This is different from what is inferred from the 'Biolog area 120h' graph (Figure 2.5b). Bacteria in these samples were able to degrade more substrates than in surface samples. The CV shows that this finding was reproducible. The reciprocal Simpson index (176) was 25.6 for A11 and 20.7 for G11 and, hence, significantly higher than the average of the subsurface samples (samples 5-10, A=13.6, D=11.4, G=9.07) and the surface samples (samples 1-3, A=15.34, D=14.53, G=16.4). It is remarkable that samples exposed to highest hydrocarbon doses are clustering together in the RDA plot since distinctive grouping of these exposed samples with PCA did not occur. The kerosene vapor concentration of these samples in the third quadrant was higher than 10 g m^{-3} at the sample day. The distance from the origin to the data point for day 23 is longer than to the point of day 80. This may be indicative of a higher influence of the contamination on functional diversity after 3 weeks. Other authors reported on distinction due to hydrocarbon contamination but in most cases they applied higher oil concentrations (126). Bundy and coworkers (28) incubated three different soils with diesel oil for 103 days in jar microcosm experiments. By performing PCA on Biolog™ GN data, they found that contaminated samples were clearly separated from uncontaminated samples, but that controls were also altered.

Biolog™ plates have in the last few years become a widely used tool in soil microbiology and they are generally considered as a sensitive tool to fingerprint communities (31, 61, 126), however, the method for analyzing the data is still open to debate. In most studies the classical GN2 plates with their 95 substrates are used (125). The reduction of substrates to 31 by using Biolog™ EcoPlates proved to be useful in our study because more repetitions could be made on one plate and the weak pattern reproducibility in the samples at depths around -1.75 and -2.90 m became apparent. This weak reproducibility as reported in the coefficient of variance in Table 2.3 occurred at all sampling times and was probably not a methodic artifact but was due to the low microbial activity. A more sensitive tetrazolium dye in Biolog™ EcoPlates would probably increase the reproducibility. However, RDA based on Biolog™ EcoPlates data allowed the distinction between major influences on the community structure. But we should not forget that 57.3 %, of the variance in the 'Biolog area 120h' data remained unexplained with our measured variables and the grouping was hence influenced by unknown factors. These factors might be

related to either the whole extraction method or the data analysis. For further studies, a CLPP method that does not require extraction of microorganisms from soil would be welcome.

2.5 Acknowledgements

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CHAPTER 3

Selective enumeration of volatile hydrocarbon-degrading bacteria in microtiter plates by a novel most probable number procedure

Karin Kaufmann, Hauke Harms and Patrick Höhener

3.1 Introduction

Enumeration of oil-degrading bacteria usually involves growth on a medium that contains crude oil or refined petroleum products as the selective substrate (27, 142, 162). Methods that use the complex petroleum cannot distinguish between degraders of different fractions. However, it may be desirable to distinguish between degraders of more recalcitrant compounds and easily oxidisable ones. Roubal and Atlas (133) modified the standard enumeration methods and used a radiorespirometric most probable number (MPN) procedure, in which ^{14}C-hexadecane was added to crude oil to estimate alkane degraders. Wrenn and Venosa (171) inoculated a hydrocarbon degrading population to a medium containing *n*-hexadecane and single polycyclic aromatic hydrocarbons (PAHs) to obtain separate estimates of microbes feeding on alkanes and aromatics. They used iodonitrotetrazolium violet (INT) for *n*-hexadecane degradation or coloration by metabolites as indicators for PAH degradation. Johnsen *et al.* (90) improved this method by using the sensitive and water-soluble tetrazolium WST-1 (sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) as indicator for PAH degradation.

WST-1 is the water-soluble form of INT (89). The sensitivity of the method was further increased when WST-1 and easily degradable, electron flow boosting carbon substrates, were simultaneously added to the hydrocarbon pre-incubated cultures. None of the mentioned methods uses highly volatile substrates, such as toluene or the carcinogenic benzene, although rapid degradation of these nocuous hydrocarbons after a petroleum spill is of high interest. A sensitive MPN method designed for volatile organic compounds may elucidate the degradation progress of such compounds in soils.

The MPN method requires a large number of dilutions in several repetitions. Because of the need for rapid and cheap methods, miniaturized systems have been proposed. However, to dose highly soluble compounds in non-toxic quantities to very small sample volumes is a challenge. Becker and Dott (16) applied hydrocarbons via soaked cardboards to bacteria on agar plates. A widely used method in biotechnology to apply toxic or poorly available substances as substrates to bioreactors is the binary liquid phase system (42, 52). The toxic or poorly water soluble compound is dissolved in a carrier phase which is immiscible with the bacteria-accommodating aqueous phase (46). The substrate concentration in the aqueous phase is mainly determined by the mole fraction of the compound in the carrier phase (see Appendix I). The criteria to consider for the selection of an appropriate carrier phase in a two liquid phase system are: biocompatibility, high solubilization capacity for the substrate, immiscibility with water, and non-biodegradability (42). Hubert *et al.* (84) used inexpensive vacuum pump oil as controlling compound and toluene as substrate, whereas Mayer *et al.* (109) preferred toluene dissolved in silicon oil. Evans *et al.* (56) investigated anaerobic toluene degradation applying a binary system with *n*-hexadecane as carrier phase. The advantage of this latter system is, that air-hexadecane partitioning coefficients ($K_{air\text{-}hex}$) are available (1) and the concentration of the compound in the gaseous phase can be calculated. *n*-Hexadecane is relatively easily degradable under aerobic conditions and was therefore unsuitable for our study. A compound with similar physical properties as *n*-hexadecane, which is less degradable by soil microorganisms, is 2,2,4,4,6,8,8-heptamethylnonane (HMN). This carrier resists to degradation by single strain experiments (170) but also to mixed microbial cultures (64, 116) because of the tertiary and quaternary methyl groups in the molecule (136). Only Rontani and Giusti (131) found a marine consortium and Katsivela *et al.* (93) isolated three strains from hydrocarbon sludge amended soil that efficiently degraded HMN.

The aim of this study was to develop and apply an MPN-method, which allows to estimate numbers of degraders of volatile organic compounds. The principle of the applied MPN-method was as follows: Soil extracts were inoculated in high-density polypropylene (PP) microtiter plates containing a mineral medium and an organic phase (HMN and substrate). The plates were immediately sealed and incubated for one week. The tetrazolium dye

WST-1 was added after incubation to assess the metabolic potential of the cells in each well. The MPN was calculated based on the formazan-colored wells. The method was evaluated in several small experiments with artificially contaminated soil. We applied the method for separately estimating the population densities of bacteria degrading either straight, branched or cyclic alkanes or aromatics. The hypotheses were 1) that we would detect more hydrocarbon degrading bacteria in a contaminated soil than in its pristine counterpart, and 2) that a soil incubated with a single hydrocarbon develops a bacterial community composed of more specialists degrading this specific compound, than a community, which has never been in contact with this hydrocarbon. A secondary aim was to test the compatibility of PP-plates with volatile hydrocarbons. A single hydrocarbon was added to a well and its diffusion into neighbouring wells was measured. This procedure, which uses 96-well microtiter plates, would be convenient for field use, because its material requirements are low, inoculation plates can easily be stored and transported, and positive wells can be identified visually.

3.2 Materials and methods

3.2.1 Vadose zone models

The alluvial sand used in this study was obtained from Sagrave SA, Lausanne, Switzerland who extracted it from Lake Geneva in the vicinity of the Rhone river delta. The sand was sieved (<2 mm) and stored at 22°C in the dark for several weeks until it was used. It had the following grain size distribution: < 2 mm: 100 % of weight, < 1 mm: 87.5 %, < 0.5 mm: 56.0 %, < 0.25 mm: 21.3 %, < 0.1 mm: 1.9 % and < 0.063 mm: 0.9 %. The organic matter content measured by TOC analysis was 2 g C kg^{-1} dry weight. The microbial population in this sand was indigenous and no nutrients were added at any stage of the experiments. The gravimetric moisture content was adjusted to 5.5 % with sterile deionized water at least one week before an experiment started. The WST-MPN method was also applied on the soil from the field experiment described in Chapter 2. It was sampled in the following depths and horizontal distances from the source, respectively: D4=1.18/ 0.75 m, E4=1.08/2 m, and F4=1.09/10 m (Figure 2.1).

3.2.2 Hydrocarbons

The kerosene composition that was used for pre-exposure was described earlier (123) and corresponds with minor weight changes to the kerosene of Table 4.2. It is composed of typical kerosene compounds with their respective weight fractions and the antiknock additive MTBE. The chosen substrates and HMN for plate incubations were all of high purity grade (Fluka, Buchs, Switzerland, only HMN was obtained from Sigma-Aldrich,

Buchs Switzerland) and represent the *n*-alkanes, branched alkanes, cycloalkanes and aromatic hydrocarbons.

3.2.3 General WST-MPN plate setup

The WST-MPN system consists of a 96 deep-well microtiter plate (Polypropylene Assay Blocks, Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands). We added to each well 100 µl mineral medium and bacteria, about 30 µl HMN-hydrocarbon mixture (for details see the experiment description) and 10 µl WST-1 after 7 days incubation, 140 µl in total. The medium was composed according to mean soil solution concentrations of nutrients (135), with the exception of phosphate which served as buffer. It consists after addition of the bacterial extract of: 2 mM (493 mg l^{-1}) $MgSO_4 \cdot 7\ H_2O$, 3 mM (240 mg l^{-1}) NH_4NO_3, 0.3 mM (70.8 mg l^{-1}) $Ca(NO_3)_2 \cdot 4\ H_2O$, 5 mM (680 mg l^{-1}) KH_2PO_4, 15 mM (2670 mg l^{-1}) $Na_2HPO_4 \cdot 2\ H_2O$ and trace element solution according to (137). The medium was adjusted to a pH of 7.2. Soil bacteria were extracted as described in Chapter 2. Particles were sedimented for 10 min and 10 ml of the supernatants transferred in new vials. They were 1:9 diluted with sterile deionised water on a separate plate. An MPN-matrix of 3 × 8 dilutions of all soil extracts was exposed to pure HMN or a mixture of a hydrocarbon substrate with HMN. Only one hydrocarbon was applied per plate to avoid cross contamination by diffusion from one well to the other. Plates were closed with lids, sealing every well separately, enveloped in air-tight plastic bags and incubated for 7 days. Ten µl of the sensitive WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) were added to each well at day 7. Subsequently the plates were vigorously shaken for 1 minute and incubated again. Since deep-well plates did not fit into the plate reader they were scanned with a flat-bed scanner (UMAX, Umax Data Systems Inc., Hsinchu, Taiwan) and dark yellow colored wells were counted by eye. MPNs were calculated according to (85), using the solver function of Microsoft® Excel 2000.

3.2.4 Microbial community changes due to kerosene contamination in Værløse soil and alluvial sand

Cell numbers in soil samples from the Værløse field site (day 23) at source depth (Figure 2.1) were estimated with the WST-MPN method. The plates were prepared as described above with the following specifications: The same soil extracts as obtained for inoculation of Biolog™ plates in Chapter 2 were used to inoculate WST-MPN plates. The hydrocarbons *n*-dodecane, toluene, isooctane or methyl-cyclohexane (MCH) were mixed with HMN (ratio 1:4) and 30 µl were added to each well. WST-1 (10 µl) was added after 7 days. Sixteen hours after WST-1 addition and incubation at 22°C, we added 20 µl sterile

filtered glucose (serving as electron donor) to a final concentration of 3.3 mM to all wells in order to boost activity, since some plates did not show any activity.

The alluvial sand was exposed to the kerosene described in (123). Samples of 50 g wet sand were incubated in a desiccator, which contained one beaker filled with 10 ml kerosene and a second one with water, at 22°C. Samples for enumerating total cell numbers were taken every 24 hours for the first five days and again at 912 hours after incubation. Microscopic slides were prepared as described in Chapter 2 but using Sybr Green II dye (79) instead of DAPI. Soil bacteria were extracted to inoculate WST-MPN plates at time 0 and after 72 hours incubation. They were treated as the Værløse soil described above also using the same substrates in the same quantities. After 7 days incubation at 22°C, 10 µl WST-1 were added and scanned after 3.5 hours. In wells where no obvious tetrazolium reduction occurred, glucose to a final concentration of 5 mM was added after 3.5 hours already.

3.2.5 Microbial community changes in alluvial sand due to single hydrocarbon contamination

Pre-exposure

150 g of wet alluvial sand was exposed to single and mixed kerosene hydrocarbons for 15 days at 25°C in the dark in specially designed 1 l Erlenmeyer flasks. The air-tight flasks were equipped with two gas sampling ports at the bottom and in the upper part of the flask. A test tube that was inserted in the lid and had an opening to the interior of the flask contained the volatile substrates: toluene/HMN, *n*-octane/HMN, MCH/HMN, isooctane/HMN, or a mixture of all four. HMN was mixed with the substances in order to achieve an initial equilibrium soil water content of 0.0378 mM carbon, being equivalent to the *n*-octane-C water solubility. Volume calculations for single substrates mixed with HMN were carried out as described in the . Every two days the presence of hydrocarbons in the headspace was analytically analyzed. If a compound was not detected anymore, the substrate source was replaced by opening only the reservoir test tube. This was hardly affecting the immediately before and afterwards measured CO_2 and O_2 concentrations (Figure 3.1).

The gas concentrations of volatile organic compounds in Erlenmeyer flasks were analyzed by injecting 50-100 µl of gas into an HP-6890 Series Gas Chromatograph (Agilent Technologies, USA), equipped with an HP-5 capillary column (33 m · 0.32 mm) and FID, using gas-tight syringes with PTFE plungers. The injector was heated to 200ºC and the split ratio set to 100. The column temperature was held at 30ºC for 5 min, then increased

to 80ºC at a rate of 10ºC min^{-1}, followed by heating at 5ºC min^{-1} to 120ºC, and then held constant for 6 min. Carrier gas was helium at a flow rate of 2 ml min^{-1}. Partial pressures of the WST-MPN incubation of CO_2 and O_2 were analyzed by injecting 100 µl of gas into a GC-8AIT gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with two PORAPAK Q columns (3 m · 1.6 mm) and a thermal conductivity detector (TCD) operated at 55°C and using N_2 as carrier gas. Dilutions of pure CO_2 and O_2 were used for calibration.

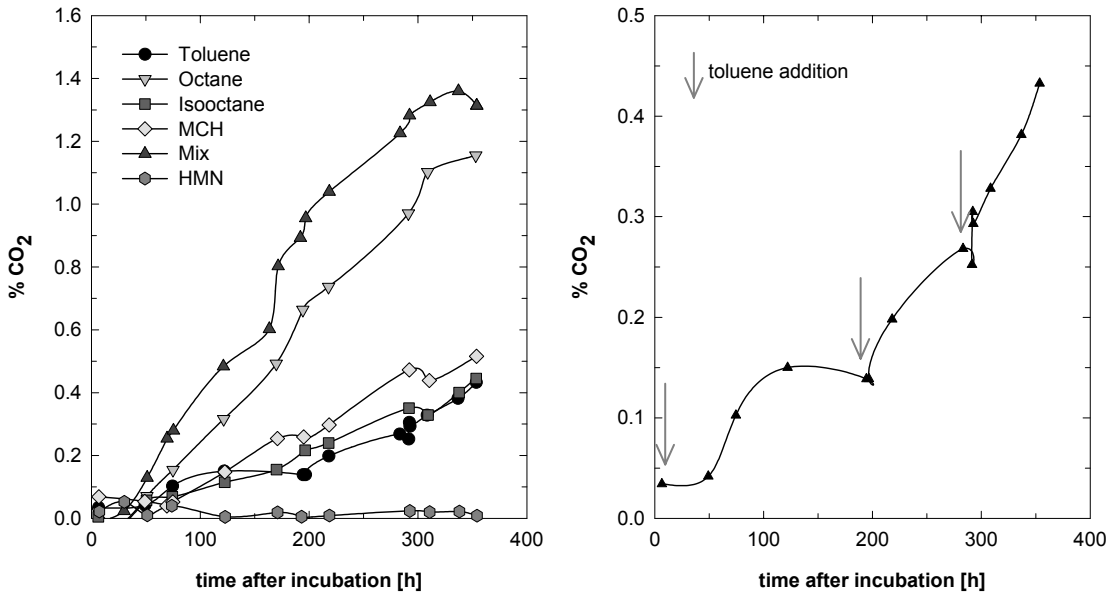

Figure 3.1: Left chart: CO_2 production during pre-exposure of sand to individual hydrocarbons. Right chart: CO_2 production as direct response to toluene addition (arrows).

WST-MPN plates, total counts and CLPP with Biolog™ EcoPlates

WST-MPN plates were prepared with 8 dilution levels over 8 orders of magnitude. The plates were amended with the same single substrates as used for pre-exposure: either *n*-octane, toluene, isooctane, or MCH were mixed with HMN. Between 32 and 51 µl of these hydrocarbon mixtures were pipetted into the wells to give the same concentrations in the aqueous medium as in the soil water during pre-exposure. Sand-extracts of all different pre-exposure treatments were inoculated on plates with the four single substrates *n*-octane, isooctane, toluene and MCH. The equations given in the Appendix I were applied to calculate the concentrations. After 3.5 hours the cells were activated by addition of 12 µl glucose to a final concentration of 5 mM (90). The plates were scanned once every two hours.

Total counts, inoculation, incubation and measurement of Biolog™ plates were performed as described in Chapter 2. To statistically analyze the Biolog™ plate results the areas under the curve between 0 and 140 hours were calculated with Riemann's sums (110) and used for data analysis.

3.2.6 Data analysis

The Biolog™ plate data matrix consisted of 31 variables on 18 samples (three replicates of 6 different incubations). To perform a discriminant analysis (DA) on those data, we reduced the number of variables by a PCA with a covariance matrix. Four principal components were introduced into S-Plus 2000 Professional Release 1 (MathSoft Inc., Cambridge, USA) for a DA. The significance of the Mahalanobis distances was tested with Hotelling's T^2 test. No Bonferroni adjustment for multiple statistical tests was applied due to reasons described in (115). The significance of differences in cell numbers after the incubation was tested with a one-way ANOVA.

3.2.7 Hydrocarbon diffusion in polypropylene (PP) plates

The 1.2 ml 96 well-plates and the lids used for the WST-MPN experiments were made from HD polypropylene (PP) and manufactured by Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands.

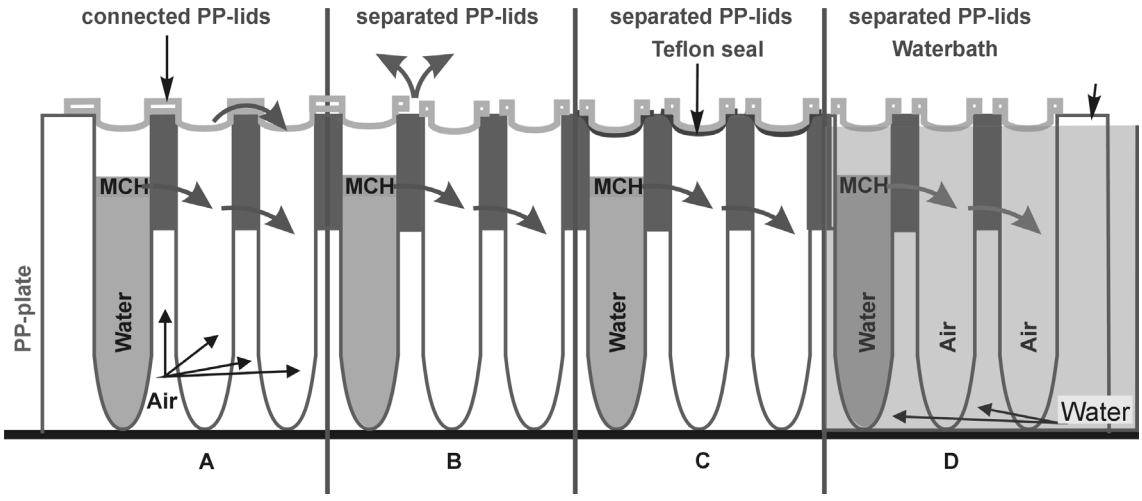

Figure 3.2: Hydrocarbon diffusion between neighbouring wells on PP plates was measured. Four set-ups were tested to minimize these cross contaminations: A) standard plates closed with standard lids, B) standard plates closed with lid strips, leaving a gap between two rows, C) like B) but a PTFE seal was added to shield the lid, D) the space between vials was filled with water by incubating the plate in a water bath.

PP is chemically more resistant but less gas-tight than the usually used polystyrene plates (information from the manufacturer). Four different plate set-ups, shown in Figure 3.2, were tested to find the combination causing least hydrocarbon cross-contamination. Therefore 100 µl water, 30 µl HMN and 5 µl methyl-cyclohexane (MCH) were added to a well and closed by PP lids.

Gas was sampled in wells from the same row as the MCH amended one. The lids were purchased as one knobbed piece sealing all 96 wells. For experiments B-D (Figure 3.2) the lid was cut in rows to create a gap between lids of the analyzed wells. PTFE seal was used to protect the PP and to decrease absorption of hydrocarbons (Figure 3.2, C). If plates are standing on a flat surface, air is enclosed between the PP plate walls and this surface. To check whether hydrocarbons diffuse into other wells via this air space, it was filled with water (Figure 3.2, D), which would then reduce diffusion from one well to the other. Hydrocarbons in the headspace were analyzed as described earlier.

3.3 Results

3.3.1 Microbial community changes upon kerosene contamination in Værløse soil and alluvial sand

The alluvial sand has been observed to efficiently degrade hydrocarbons after a short lag phase (123). The incubations in the desiccator show that cell numbers increase about 3 times in presence of kerosene (Figure 3.3).

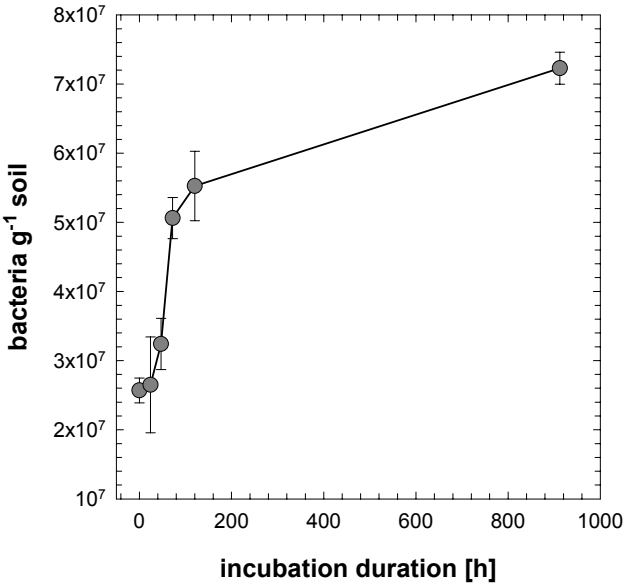

Figure 3.3: Time dependent total cell number increase in alluvial sand exposed to a kerosene-saturated atmosphere (from (79)).

In wells with high activity the tetrazolium dye was efficiently reduced to the yellow/orange formazan within 3.5 hours. The development of a signal within 3.5 hours corresponds to the manufacturer's recommendation to measure after 0.5 to 4 hours after WST-1 addition. For all substrates other than isooctane a signal was obtained. Addition of glucose did not produce further coloration after another 3.5 hours incubation in isooctane wells.

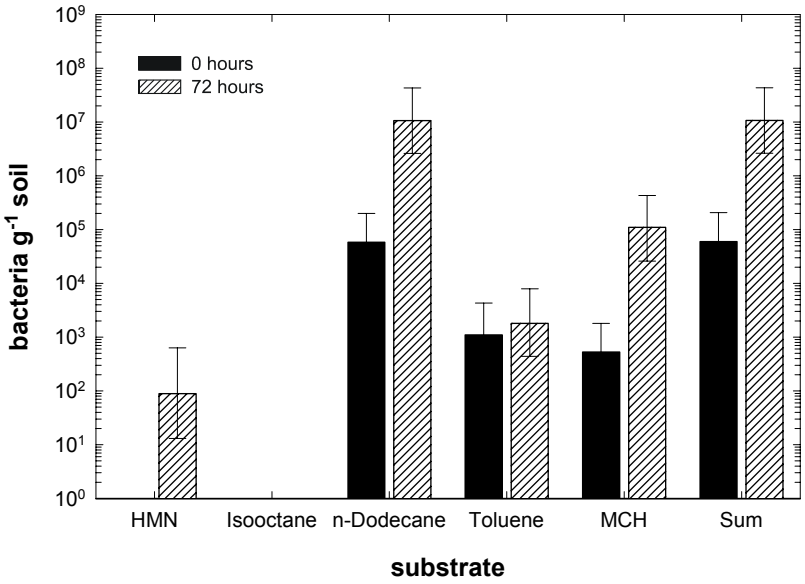

Figure 3.4: MPN from alluvial sand that had been exposed for 0 and 72 hours to a kerosene-saturated atmosphere. HMN, isooctane, *n*-dodecane, toluene, and MCH degraders are added up in the bars referred to as 'Sum'. Error bars represent the 95 % confidence interval.

Estimation of specific substrate degraders with the WST-MPN plates revealed a 100 fold increase in bacteria able to degrade hydrocarbons within the first 72 hours of incubation. The totality of detected hydrocarbon degraders, referred to as 'Sum' in Figure 3.4, accounted for 0.23 % of total cells (DAPI counts) in the pristine soil and 21 % in the contaminated soil. The main part of the bacterial community was composed of *n*-dodecane degraders (Figure 3.4). Isooctane degraders were neither detected in the pristine soil nor in the soil incubated for 72 hours.

When the same method was applied to the day-23 Værløse sand, we observed that the samples that were taken closer to the source resulted in weaker formazan production (Figure 3.5). Stimulation with glucose led to a stronger coloration on plate E4 (2 m), but did not significantly improve the coloration on plate D4 (0.75 m). By microscopically examining the bacterial numbers of corresponding wells, we detected more cells in wells on

plate D4 than F4, although only wells on F4 turned clearly dark yellow/orange. We further observed that strongest coloration did not always occur in wells with highest cell densities as would be expected, but in wells with intermediate cell densities (Figure 3.5, plate E4). As it was difficult to accurately estimate all cell numbers in these samples no numbers are given.

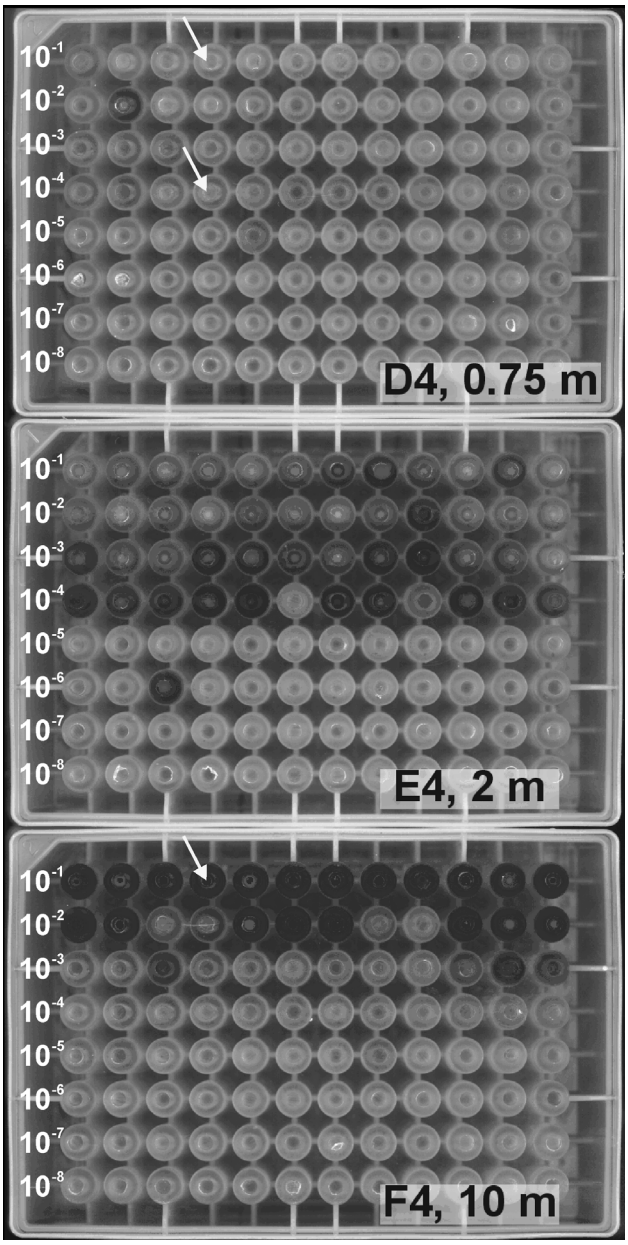

Figure 3.5: Scan of MPN plates 15 hours after WST-1 addition, inoculated with soil extracts from Værløse soil exposed to kerosene vapors for 23 days. The yellow/orange formazan appears black in this picture. Sample names and horizontal distances to the kerosene source correspond to the scheme in Chapter 2 (Figure 2.1). Rows are labeled with the dilutions and arrows indicate the microscopically examined wells.

3.3.2 Microbial community changes due to singular hydrocarbon contamination

Activity in incubation flasks

Figure 3.1 shows the development of CO_2 in the pre-exposure flasks as function of the incubation time. Little CO_2 was developed with HMN as sole source of carbon. Highest CO_2 yields were obtained by the bacterial community exposed to the mixture of all four substrates, followed by the one grown on *n*-octane. The degradation in the MCH containing flask was slightly higher than with isooctane and toluene as substrates. However, the CO_2 concentration of 0.516 % in the MCH pre-exposure flask after 350 hours incubation was about one third of the concentration in the mixture flask. Toluene induced fast CO_2 production in the beginning, but headspace analyzes revealed that the carbon source was depleted within a short period. Toluene as the most water-soluble substrate, was only applied in small amounts to achieve a water concentration of 0.0378 mM (3.25 µl toluene/ 10 ml HMN). The CO_2 production decreased but increased again immediately after new toluene addition (Figure 3.1).

Cell number increase due to pre-exposure

Cell numbers and CO_2 concentration were not correlated after 350 hours of pre-exposure. The numbers of bacteria increased in all flasks compared to the inital values, although not in all cases significantly.

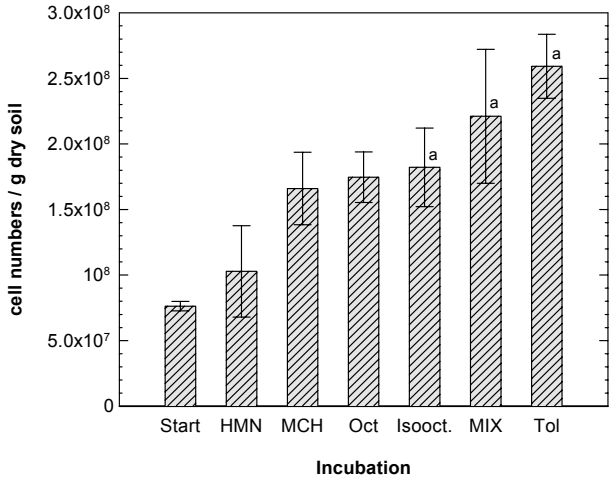

Figure 3.6: Cell numbers in the alluvial sand at the start and after 350 hours incubation. Bars represent the standard error, 'a' indicates the incubations in which cell numbers significantly increased.

The highest increase occurred in the toluene-fed community, which quadrupled. Bacterial numbers in the mixture flask tripled. Both communities significantly increased in cell numbers compared to bacteria in the pristine and the HMN sand. Although the organisms in the *n*-octane flasks were very active, the cell number increased not more than in the MCH and isooctane flask (Figure 3.6).

Hydrocarbon degrader estimation by WST-MPN

In the wells of the *n*-octane plate an intensive signal developed within 30 minutes after WST-1 addition. The tetrazolium reduction on other plates was weak and glucose was added to boost its reduction. The colour intensity of the slightly yellowish wells increased within the next 3.5 hours, but no additional wells turned yellow. MPN were calculated based on the number of positive wells 3.5 hours after glucose addition. Table 3.1 shows the cell number estimates of hydrocarbon degrading organisms and the corresponding 95 % and 5 % confidence interval of all combinations of different pre-exposure treatments and hydrocarbon substrates provided on WST-MPN plates.

Table 3.1: Cell numbers per g dry sand and the corresponding 95 % confidence intervals of incubations with single hydrocarbons, estimated with the WST-MPN method. Plate substrates are arranged in columns and pre-exposure substrates in rows.

Plate	Blank			Isooctane			Octane			Toluene			MCH		
Pre-exp	MPN	UL[a]	LL[b]	MPN	UL[a]	LL[b]	MPN	UL[a]	LL[b]	MPN	UL[a]	LL[b]	MPN	UL[a]	LL[b]
Mix	118	414	34	0	0	0	459330	1836350	114890	138	1218	16	1797	6930	466
n-Octane	0	0	0	46	185	11	55760	210400	14780	179	1260	26	459	1835	115
MCH	0	0	0	45	183	11	4589	18350	1147	45	1710	1	1336	4763	375
Isooct	0	0	0	0	0	0	571	2141	152	15	7697	0	0	0	0
Toluene	0	0	0	99	344	29	3484	14970	811	218	1320	36	212	863	52
HMN	140	502	39	90	313	26	1378	4944	384	95	1244	7	121	424	34

a upper limit of the 95% confidence interval
b lower limit of the 95% confidence interval

The MPN results are examined in view of the hypothesis that communities reflect their pre-conditioning substrates in having high numbers of specialists able to degrade this specific substrate: this was true on n-octane plates, since the highest cell numbers were obtained from samples pre-exposed with n-octane and the n-octane containing mixture. Preconditioned samples showed also on the MCH-plate higher cell numbers than samples, which were pre-exposed to other substrates. However, the estimated numbers of MCH-degraders were very low. Also, very low microbial numbers were observed on the toluene

plate, although the toluene pre-exposed community showed the highest number. Hardly any bacteria were detected on isooctane plates. Johnsen et al. (90) calculated the MPN detection limit by assuming one wrongly positive well at the lowest dilution. One positive well with the dilution factor used in this experiment results in 18 bacteria (UL 95 % = 125, LL 95 % = 3). To be 95 % sure that the number is superior to the detection limit, it would be set at 125 cells. All calculated numbers on isooctane were below the detection limit.

CLPP with Biolog™ EcoPlates

Applying a discriminant analysis on areas under Biolog™ absorbance curves allows distinguishing between the treatments in the pre-exposure flasks (Figure 3.7). The significance of distances between groups was tested with Hotelling's T^2 test, the results are listed in Table 3.2. The HMN-incubation only differs significantly from the *n*-octane pre-exposed community. The mixture and *n*-octane pre-exposure seemed to induce similar effects in the soil community since there is no discrimination between the two of them. But both are separated from the toluene, isooctane, and MCH pre-exposed samples by the DA factor 1. Factor 1, factor 2 and factor 3 explained 66.7 %, 27.4 % and 4.6 % of variation, respectively. The model was overall significant ($p<0.001$).

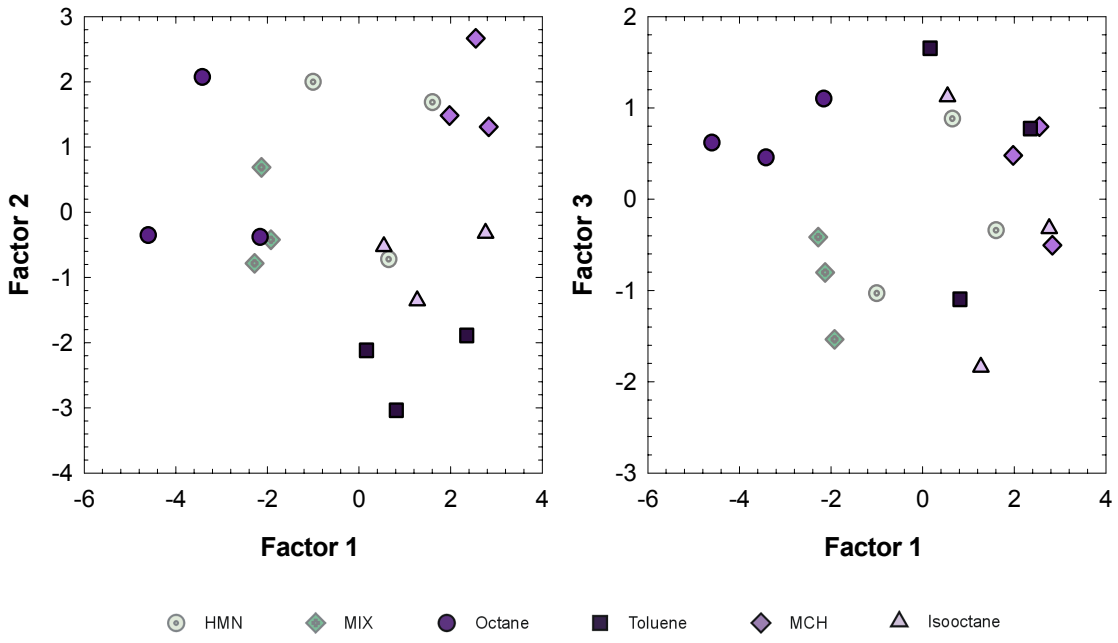

Figure 3.7: Discriminant analysis of results of Biolog™ EcoPlates inoculated with cell extracts of the samples pre-exposed to single or mixed hydrocarbons.

3.3.3 Hydrocarbon diffusion in PP plates

High density polypropylene (PP) plates are more resistant against aggressive chemicals than polystyrene plates but still not as gas tight and inert as glass. While toluene completely deteriorated polystyrene plates in preliminary experiments (results not shown), no immediate effect was visible with pure toluene application on PP plates. Nevertheless, PP plates were used as cost effective alternative to glass plates. Four possible combinations of sealing the plates and preventing gas to diffuse from one well to the other are illustrated in Figure 3.2. MCH was added into one well of the plate. Diffusion was determined by measuring the MCH concentration in the air of the wells in the same row.

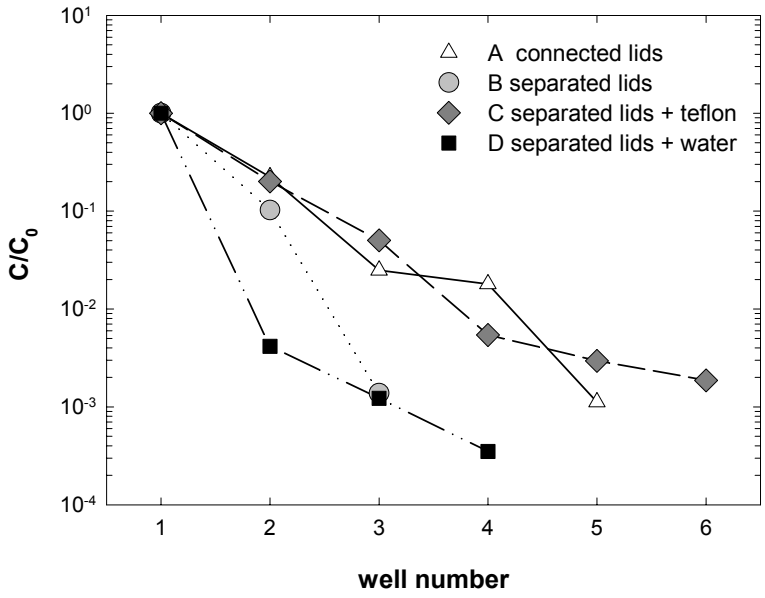

Figure 3.8: Ratio of MCH concentration as function of the distance to the originally amended well (well No 1).

In Figure 3.8 it is expressed as ratio of the concentration in the 'amended' well, denoted as well No 1. In the well next to the amended one (No 2) we measured concentrations of 20 % with connected (A) and separated but PTFE shielded lids (C), 10 % with separated lids (B), 0.4 % with separated lids but a water filled space between wells (D). The gradient from one well to the next is steeper with separated lid rows than with connected ones. Hydrocarbon loss in wells with MCH was measured as a function of time on a plate covered with separated lid rows. Loss occurred linearly, the MCH concentration decreased after 150 hours incubation to less than half of the initial concentration.

3.4 Discussion

The experiments described in this chapter aimed at developing a physiological method to obtain better insight into changing microbial numbers in contaminated sites. The gained information should be specific for the contamination and, if regularly applied, show changes in the bacterial community as a function of time. The hypothesis for the experiments described here was, that soils with hydrocarbon exposure in their history have greater hydrocarbon oxidizing activity than soils without a contamination history, an observation already described in (32). This hypothesis was confirmed with the WST-MPN method. An alluvial sand, which showed degradation activity in earlier experiments (123) was chosen as the main study object and incubated in presence of volatile hydrocarbons. Immediate increase of the CO_2 concentration after toluene application suggested that the CO_2-release was linked to the hydrocarbon mineralization. HMN did not induce any CO_2 production higher than atmospheric concentrations, which is a premise for a carrier compound.

Cell numbers increased in presence of single or mixed substrates within few hours. The cell density in soil extracts was high enough to induce good reproducibility in the reduction pattern on Biolog™ EcoPlates. With the Biolog™ assay we could distinguish between the pre-exposure treatments and show that community changes are visible on the physiological level. Soil communities pre-exposed to *n*-octane and hydrocarbon mixture developed in the same direction and did not significantly differ. This is taken as an indication for a dominant influence of the easily oxidizable *n*-octane. Alkane degraders are most abundant in a lot of environments and may mask the presence of other degraders (171). *n*-Dodecane and *n*-octane dominance in the mixture community is reflected on the WST-MPN plates. In the time course experiment as well as in the single carbon experiment the two alkanes *n*-dodecane and *n*-octane yielded highest cell numbers. Isooctane differed least from the blank in the Biolog™ assay (Table 3.2), which is again reflected in the low cell numbers in both experiments.

On the WST-MPN even the isooctane plates with the densest inoculum were hardly yellow, and the signal did not improve after a 3.5 hours boost with glucose. The CO_2 that is produced during the pre-exposure with isooctane may mainly come from cell maintenance activity but not from growth. Glucose is supposed to be degradable by most organisms (11, 44) and was used to accelerate formazan deposition in other studies (150). However, Johnsen *et al.* (90) report of a mixture of pyruvate, succinate, and glucose to be the most effective boosting substrate for PAH degraders, and this mixture might have increased the sensitivity of our WST-MPN method. The signal obtained with tetrazolium is depending on the reducing potential of organisms. Cells grown on one substrate may not

deposit as much formazan as the same number of cells grown on another substrate. Wrenn and Venosa (171) report that *n*-hexadecane-grown cells reduce 3 times more INT than cells grown on fuel oil No 2. This is in accordance with our observation that the color intensity was strongest on *n*-alkane plates, even at high dilutions.

Table 3.2: Pre-exposure lead to significantly ($p<0.05$) distinguishable groups with a DA applied on Biolog^{TM} EcoPlate results.

Significant		Not significant	
Pre-exposure	p	Pre-exposure	p
HMN-Oct	0.030	HMN-Isooct	0.377
Isooct-Mix	0.038	HMN-MCH	0.282
Isooct-Oct	0.006	HMN-Mix	0.124
MCH-Mix	0.006	HMN-Tol	0.057
MCH-Oct	0.002	Isooct-MCH	0.135
MCH-Tol	0.017	Isooct-Tol	0.429
Mix-Tol	0.024	Mix-Oct	0.316
Oct-Tol	0.005		

We observed a high increase in cell numbers in the sand pre-exposed to toluene, although the substrate was limiting. Little tetrazolium, however, was reduced in toluene-amended wells and MPNs were very low, lower than numbers estimated in the time course experiment. The toluene concentration applied on plates of the single hydrocarbon experiment was equal to the *n*-octane solubility and was smaller than in the time course experiment. Since toluene is the most water-soluble of the four compounds, the added volume and the reservoir in the carrier phase HMN was smaller than for other substances, and may have been depleted.

The hydrocarbon diffusion experiment shows that the plates are not completely tight. Even though the relative loss per well is smaller in a completely filled plate than if only one well is substrate amended, the concentration may have fallen below a critical threshold, so that the MPNs from the toluene plate of the single substrate experiment have to be considered with caution. The measurement of MCH in wells next to the only amended one showed that the wells are not isolated from each other. Two ways for hydrocarbons to travel from one well to the other can be imagined: 1) via the airspace between the plate walls and the underground surface or 2) if the lids are not completely tight, between lid and plate. Concentrations in neighbouring wells decrease by separating lid rows and creating a gap, where vapors could escape instead of diffusing in the surrounding wells. This

suggests that the lids were not completely tight or that MCH diffused between lid and plate into other wells. Adding a PTFE seal below the lid rows increased MCH concentrations in the neighbouring wells again. Hence, if well lids are tightened and hydrocarbon concentrations increase, MCH must diffuse from the bottom into the wells. This was tried to stop by filling the void between the walls of neighbouring wells with water. Here the concentration decreased at least in the closest well to the amended one. PP plates are more resistant to chemicals than polystyrene plates but less gas tight, and their physical and chemical properties are not suitable for our purpose. While cross contamination can be avoided when only one hydrocarbon per plate is applied, it will be difficult to prevent hydrocarbon loss.

After the first encouraging results with WST-MPN plates they were applied in the GRACOS field experiment. Strong tetrazolium reduction occurred in the plate that was inoculated with soil sampled 10 m from the contaminant source (Figure 3.5). However, a low signal was observed on the plate inoculated with soil from 0.7 m distance. This observation was a drawback for the method. The increased CO_2 concentrations in the field (see Chapter 2) show that an active microbial community is present in the soil, especially at 0.7 m. The microscopically observed bacterial numbers, although the cells are not surely viable, are higher in wells of the not colored D4 than the colored E4 plate from the Værløse filled experiment (Figure 3.5). Further, wells with intermediate dilutions colored faster than wells at low dilutions. The same phenomenon was observed by Johnsen *et al.* (90) in MPN tests with WST-1 and PAH degraders. They attributed low signals on low dilution levels to substrate depletion and toxic metabolite accumulation in wells. Other authors (102) found negative effects of certain organic substances (such as phenazine metosulphate and methylene blue) on formazan production because they transfer electrons directly to oxygen in competition with a tetrazolium recipient (127). Water-soluble metabolites, present in the soil in increasing concentration with decreasing distance to the contaminant source, better explains the gradually decreasing formazan production as a function of the decreasing distance than metabolite accumulation due to degradation on the plates, since the whole plate was affected by low signals. This metabolite, potentially present in intermediate concentrations on E4 plates, may have been sufficiently reduced by dilution, so that formazan reduction became possible at intermediate dilutions.

The MPN technique is the only technique that has been used to date to enumerate microbes of a specific metabolic type in subsurface samples, except the nowadays widely used genetic fingerprinting methods (48). Although MPNs have to be interpreted with caution, since culturing techniques underestimate microbial cell numbers in soil and especially in subsoil, it is an easy method to get an impression of changes in the microbial community. It must be recognized that soil organisms are often clustered and the method

may estimate the numbers of physical units such as soil particles with associated organisms, or 'physiological units' (e.g microbial consortia) involved in the same process, rather than numbers of bacteria per se (48). We were able to detect more degraders of volatile organic carbon in contaminated soils than in pristine soils. Also the second hypothesis, that single hydrocarbon incubation boosted specialists for this compound, was partly confirmed. However, the method is not yet sensitive enough to detect isooctane degraders. Longer incubation may increase sensitivity but therefore a tighter deep-well plate with resistant glass vials would be required. To reduce costs and circumvent uncertainties with the tetrazolium dye, a hybrid system of the described MPN plate with the agar detection system of the MicroResp system (Chapters 4 and 5) could be imagined.

3.5 Acknowledgements

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CHAPTER 4

Soil microbial community response to hydrocarbon pollution assessed using a micro-respiration method incorporating a two-liquid phase system to control volatile substrate concentration

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4.1 Introduction

The ability of soil microbial communities to transform petroleum hydrocarbons into CO_2 is of general interest at spill sites where engineered bioremediation or enhanced natural attenuation are the main remediation options. However, traditional microbial methods to investigate hydrocarbon biodegradation in soils are time-consuming. Examining the functional diversity of hydrocarbon-degrading bacteria, Caparello and LaRock (32) found that the hydrocarbon-oxidizing potential of environmental samples reflects the hydrocarbon burden of the area and the ability of the indigenous microorganisms to utilize hydrocarbons. Pucci *et al.* (126) found significant increases in hydrocarbon-utilizing microorganisms in soils receiving hydrocarbons. Walker and Colwell (162) observed greater rates of

uptake and mineralization for bacteria in samples collected from an oil-polluted harbor than for samples from a relatively unpolluted region. A large number of microcosm studies have demonstrated sizable increases of populations of hydrocarbon-utilizing microorganisms in environmental samples exposed to petroleum hydrocarbons (79, 142).

Several processes contribute to community changes in polluted soils: When the pollution exceeds a critical level, toxic effects inhibit the most sensitive organisms. This process causes a decreased fitness of these organisms leading to out-competition by more tolerant ones (159) and, as a consequence, a loss of species diversity (23). It was stated that adaptation should not be considered as an all-or-none phenomenon, but rather as a quantitative one dependent on pre-exposure time and concentration (144). According to these observations it should be possible to distinguish two similar soils, a petroleum-contaminated one and an uncontaminated control, based on their microbial community. Community changes are manifested in cell numbers in the diversity but also in the activity.

There is a need for rapid and cheap methods to detect community changes in soils and water, that allow to simultaneously analyze a large number of repetitions. Therefore miniaturized methods have been proposed. Although not designed for environmental applications (31), Biolog^{TM} plates were widely used to distinguish between stressed or perturbed soils (24, 28, 55, 126, 172). They measure the specific dehydrogenase activity of environmental samples in presence of water-soluble substrates. Petroleum hydrocarbon specific methods have been developed either to detect degradation potential for aromatics and aliphatics (16) or to enumerate petroleum degrading bacteria (27). These methods are all either applied to water samples or to liquid extracts from soils.

Campbell *et al.* (30) have developed a miniaturized system (MicroResp) to monitor CO_2 production from soluble carbon substrates in 96 soil samples directly incubated in a multiwell plate. The system has been modified in this thesis for the use with petroleum hydrocarbons as substrates, applied in HMN as carrier phase (94). In Chapter 5 we shall demonstrate the application of a modified MicroResp system to study nutrient limitation for petroleum hydrocarbon degradation in soil. The aims of this study here were 1) to investigate the effects of pre-exposure of soil to hydrocarbons on soil DNA content and soil respiration, 2) to demonstrate the applicability of the modified MicroResp system to investigate effects on the microbial community, and 3) to demonstrate the utility of the modified MicroResp system as a tool for the identification of inhibitory concentrations of petroleum hydrocarbons.

4.2 Materials and methods

4.2.1 Experimental design

Two main experiments, referred to in the following as 'MicroResp activity experiment' and 'MicroResp inhibition experiment', were carried out. Both included two different soils, a hydrocarbon-contaminated subsurface soil and a surface soil from an agricultural area. The soils were adjusted to 40 % of their water holding capacity (WHC). One half of each soil was incubated under an atmosphere saturated with petroleum vapor, whereas the other half was incubated in the absence of petroleum hydrocarbons. The incubation at 25°C in the dark lasted 37 and 25 days for the MicroResp activity and MicroResp inhibition experiments, respectively. CO_2 concentrations in the jars were measured during the experiment and DNA contents were determined in the beginning and at the end of the pre-exposure. After incubation in the jars, the soils were filled into the MicroResp plates and incubated in the presence of single hydrocarbons. The color change, due to CO_2 production in the detection plate of the MicroResp system, was spectrophotometrically determined for the incubation periods of 0-6, 6-24 and 24-30 hours after incubation start, using for each period a new detection plate.

4.2.2 Soils

The first soil (soil AS) was a sandy soil located below a commercial petrol station in Assebroek, Belgium, which had been operating for 10 years (Table 4.1). On this site, BTEX (benzene, toluene, ethylbenzene, xylenes) and MTBE (methyl *tert*-butyl ether) concentrations between 380-1000 and 300-5400 µg/kg, respectively were previously measured in borehole samples (Gunther De Becker, pers. comm.). Experiments described in Chapter 5 have shown that this soil is nutrient deficient. The second soil (soil CWR) was an agricultural soil sampled next to Countesswells Road in Aberdeen, Scotland. Samples were taken at 10-30 cm depth, after removal of the grass layer. Both soils were sieved <2 mm and stored at 25°C until use. Soil properties were analyzed by using standard methods as described in (94) and summarized results are given in Table 4.1.

4.2.3 Pre-exposure

For both experiments, the soil moisture content was adjusted to 40 % WHC with sterile water at least one week before the experiment was started. Twelve bottling jars (1.5 l, Kilner®, Ravenhead Company Ltd., Staines, UK), equipped with butyl rubber stoppers for gas-sampling were used for incubations of six times either 160 g AS or 100 g CWR soil.

The quantities were determined according to the volume needed for MicroResp incubations.

Table 4.1: Selected soil properties of the Assebroek (AS) and Countesswells Road (CWR) soil. Concentrations are given in [mg kg^{-1}] unless indicated differently.

	Soils	
Analysis	AS	CWR
pH	7.8	5.4
Initial gravimetric water content [g g^{-1} wet soil]	0.048	0.271
Water holding capacity (WHC) [%]	14	57.5
Total carbon [g kg^{-1}]	8.3	36.9
Total organic carbon [g kg^{-1}]	6.3	35.8
Extractable P	5.2	ND
Extractable NH_4^+	1.21	ND
Extractable NO_3^-	2.77	ND
Acid extractable Mg^{2+}	143.6	78.25
Acid extractable Ca^{2+}	6880	1203
Acid extractable K^+	96.0	121.5

ND: not determined

In 3 of the 6 jars of each soil, we placed an open glass vial at the bottom, which contained an artificial petroleum hydrocarbon mixture (Table 4.2). The mixture was composed of typical gasoline and kerosene compounds and the antiknock additive MTBE. All compounds with the exception of MTBE were readily biodegradable and were already used in (123). The majority of the products were purchased from Fluka (Sigma-Aldrich Company Ltd., Dorset, UK). HMN, *n*-dodecane, *n*-octane, and isooctane were obtained from Sigma (Sigma-Aldrich Company, Dorset, UK). All products had a purity grade higher than 98 %. Soils from hydrocarbon pre-exposed samples are in the continuation denoted as ker+, whereas samples incubated without kerosene are named ker-. The jars were incubated for 37 (MicroResp-activity) or 25 days (MicroResp-inhibition) at 25°C in the dark. During pre-exposure, all jars were opened for half an hour at intervals of 5-10 days to ensure aeration and to refuel fresh petroleum hydrocarbon mixture.

4.2.4 CO_2 measurements in jars

During 24 h before sampling the soil, the CO_2 production following aeration and closure of the jars was measured. Using a gas-tight syringe, 1 ml gas of each jar was sampled. A Trace^{TM} GC (Thermo Finnigan, Milan, Italy) equipped with a 10 m silica Chrompack capillary column (Varian Ltd., Warrington, UK), and a thermal conductivity detector (TCD) was used for CO_2 measurements. Helium was the carrier gas. The injector, oven and TCD

filament temperature were held at 200°C, 60°C and 350°C, respectively during analysis. All measurements were conducted at least in triplicates.

4.2.5 DNA quantification in soils

DNA was quantified in the soils before and after pre-exposure in order to monitor changes in microbial biomass. Immediately after sampling, soil samples were therefore subdivided in 0.5 g portions and frozen at –20°C until further use. The soil DNA of three replicates of each sample was extracted by applying a modified protocol of the method described by Griffiths *et al.* (70) but using the glass bead containing Lysing Matrix E tubes for extraction, which are part of the FastDNA® SPIN Kit for Soil (BIO 101, Carlsbad, CA, USA). Subsequently, the DNA was precipitated and washed applying the last 6 steps of the FastDNA® SPIN Kit for soil. After elution with 200 µl DES-buffer the nucleic acids were stored at -20°C. DNA was roughly quantified using a BioPhotometer (Eppendorf UK Ltd., Cambridge, UK) in order to find the appropriate dilution for quantification with the more sensitive PicoGreen® dsDNA Quantitation Kit (Molecular Probes Inc., Leiden, The Netherlands). To quantify the DNA with PicoGreen® all samples were prepared as recommended by the manufacturer and measured in triplicates with a CytoFluor® Multi-well Plate Reader, series 4000 (PerSeptive Biosystems, Foster City, CA, USA), which was equipped with the CytoFluor software version 4.2. A DNA standard curve over a concentration range of 0-1000 ng ml^{-1}, was prepared with bacteriophage lambda DNA provided with the PicoGreen® kit. Data were analyzed by a two-way ANOVA using SPSS release 10.0.5 (SPSS Inc., Chicago, USA).

4.2.6 Modified MicroResp system

For this study, we used the modified MicroResp system (30) specially designed to work with volatile petroleum hydrocarbons as substrates (Figure 4.1 and 94). A Multi-Tier™ topas glass deep-well plate, purchased by Wheaton Scientific Products Ltd., Milleville, NJ, USA, served as bottom plate. The volume of each of the 96 removable vials was 1 ml. These vials were sorted by length (± 0.1 mm) in order to obtain an even surface. Quantities of 0.3 g muffled (500°C) glass beads (∅ 0.25-0.56 mm) were filled with a specially designed spoon into each well before the plates were autoclaved and subsequently dried. A standard microtiter plate (Sero-Wel, Bibby Sterilin Ltd., Statts, UK) with a sliding bottom served as filling plate. Wells that were not used or reserved to be filled later with different soils were taped. During the filling procedure the plate was shaken in order to get equal density in all wells. This allowed to fill 0.28 g CWR soil, and 0.50 g AS soil into each vial. The filling plate was precisely positioned on the deep-well plate and the soil was transferred into it by hitting the whole on a flat surface. Few remaining particles were

pushed down with a sterile syringe needle. The filled deep-well plates were stored for 72 hours in air-tight boxes containing a wet tissue and soda-lime as CO_2 trap.

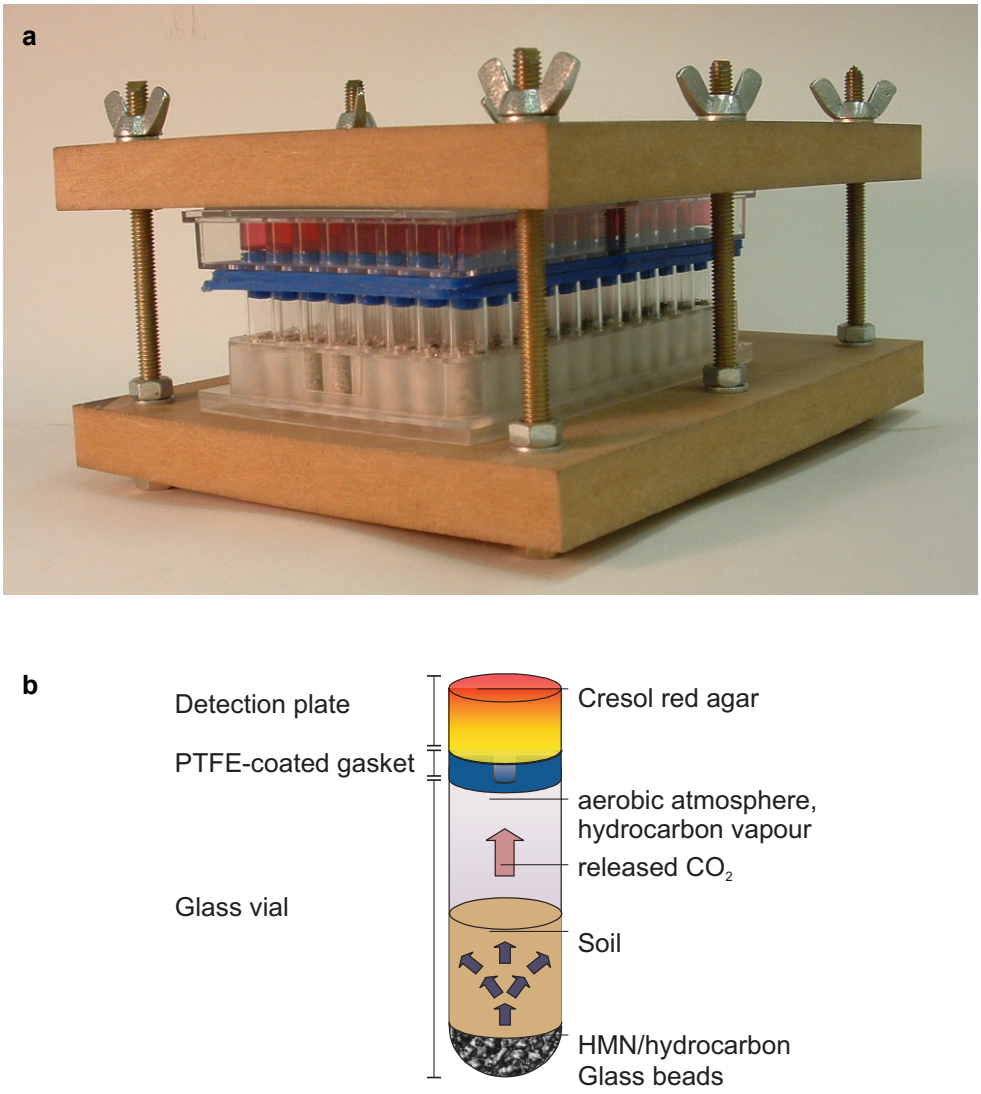

Figure 4.1: a) the MicroResp system composed of a deep-well plate, a gasket, and a detection plate containing cresol red agar. The system is hold together with a clamp. b) Schematic view of a glass vial connected to the detection (containing cresol red agar) via a silicon gasket.

Carry over of hydrocarbons was avoided by keeping different soils and pre-exposure treatments separated form each other. Before the hydrocarbon substrates were added onto the plates, vials were rearranged so that not more than two different hydrocarbons per plate were used. Substrates were applied as mixtures with HMN for reasons described below and arranged row-wise. The hydrocarbon-HMN mixtures (Table 4.2) were prepared

immediately before use. Thirty µl of these mixtures were transferred on the bottom of each well by means of a pipette equipped with a shortened pipette tip carrying a syringe needle. The filled wells were immediately closed with a strip of a microtiter plate lid. Once all the substrates were distributed into the wells, a holed PTFE layered WebSeal™ gasket (Chromacol Ltd., Herts, UK) was stuck on the vials. The system was completed with an upside down-positioned indicator plate containing in each well 200 µl cresol red agar. The system was kept gas tight with a clamp and incubated at 25°C.

One detection plate was used per incubation period and deep-well plate. Two-hundred-fifty ml cresol red agar was prepared as described in (30) containing 1.5 mM $NaHCO_3$, 100 mM KCl, 12 g l^{-1} Nobel agar, and 12 mg l^{-1} cresol red. The $NaHCO_3$ content was reduced compared to (30) in order to make the system more sensitive. The absorbance was measured at 450 and 590 nm at time 0, 6, 24 and for the activity experiment 30 hours after incubation start, using a plate reader (Biolog MicroStation™ System, Biolog Inc., Hayward, Ca, USA), which was equipped with Softmax Pro 3.1 software (Molecular Devices Corp., Sunnyvale, Ca, USA). In the first MicroResp study (30) the absorbance was evaluated at 590 nm. To calculate the absorbance difference at 450 and 590 nm was found to render the system slightly more sensitive since disappearance of purple and appearance of yellow are both included in the analysis. The results of the time 0 measurements were subtracted from respective plates to account for manufacturing divergences between wells.

4.2.7 Heptamethylnonane as carrier phase for hydrocarbon substrates

In this study, HMN was used as carrier phase. HMN was added to the pore space in the glass bead layer on the bottom of each vial and was hence not in direct contact with the soil. Transfer of hydrocarbons to soil organisms was assured via gas-phase diffusion. Using HMN as a carrier phase ensures that the hydrocarbon substrate is applied at constant and low concentration over the plate incubation duration. HMN itself has a low volatility and biodegradability. The theoretical concentrations of the hydrocarbon substrates in the air or soil water phase on the plates can be calculated by using Raoult's law. Formulas are given in the Appendix I.

The HMN reservoir contains between 92-99.9 % of the total hydrocarbon in case of apolar compounds, 2.5 % and 0.3 % of the very hydrophilic MTBE and ethanol. The bulk part of these two compounds (89 and 99 %) was dissolved in water, only <2.1 % of the apolar substrates theoretically occurred in this compartment, which consists of the soil water and the agar phase located in the detection plate. The air phase accounted for 0.1 to 7.7 % for all substrates.

In our calculations, we did not consider any sorption to glass, lid, gasket materials or the soil. The soil water concentration will be lower than the theoretically calculated values and concentrations given in this study are the maximum values, lying above real concentrations. In the following we will either use the term 'target concentrations' to make clear, that this value is a theoretical one, or give mole fractions of the substrate in the applied substrate/HMN mixture.

Table 4.2: Hydrocarbon compounds and initial target concentrations used for the different parts of the experiments.

Compound	Pre-exposure	Pre-exposure	MicroResp-activity	MicroResp-inhibition
	Art. kerosene composition (wt%)	Substrate conc. in soil water [μM]	Substrate conc. in soil water [μM]	Conc. range in soil water [μM][a]
Toluene	3.00	200	5.4	0 to 6030
m-Xylene	5.03	7.8	4.7	0 to 1480
1,2,4-TMB[b]	6.33	2.7	4.2	0 to 474
Cyclohexane	4.79	4.0	-	-
Methylcyclopentane	6.05	3.8	-	-
Methylcyclohexane	10.9	1.7	5.4	-
n-Dodecane	9.07	0.0013	3.2	-
n-Decane	17.13	0.034	3.8	-
n-Octane	8.06	0.45	4.7	-
n-Hexane	6.94	13	6.3	0 to 110
n-Pentane	3.03	24	-	0 to 534
Isooctane	15.45	3.0	-	-
MTBE	5.01	35000	7.6	-
Ethanol	-	-	-	0 to 2.2 10^6
2-Methylpentane	-	-	6.3	-

a Maximum values correspond to maximum water solubilities. References are listed in (67). The value given for ethanol was calculated from the amount soil water and ethanol present in the vial.
b 1,2,4-Trimethylbenzene
- not applied

4.2.8 Substrates and concentrations on plates

For the MicroResp activity experiment, low substrate concentrations were applied. For all substrates, aqueous phase concentrations should not exceed the concentration of the least water soluble substrate (*n*-octane-C, $C_{i\ water}^{sat}$ = 0.0378 mM) in order to make sure that all substrates occur in similar aqueous concentrations on the basis of equal carbon numbers. These concentrations are in general lower than in the initial concentrations of pre-exposure. In the MicroResp inhibition experiment, target concentration ranged over several or-

ders of magnitude. The upper concentration limit in MicroResp inhibition is given by the water solubility of the pure substance. The substrates and their target concentrations used on plates in both experiments are listed in Table 4.2. Blanks without any substrate and applications of pure HMN are not mentioned in Table 4.2.

4.2.9 Calibration of detection plates

CO_2 production in vials and CO_2 diffusion into the cresol red agar is a dynamic process, which leads to a pH and therefore a color gradient within the detection wells. Consequently, the color change was calibrated with carbon dioxide under the same dynamic conditions, providing toluene as substrate to bacteria of both soils in gas-tight bottles. CO_2 in the headspace was measured every two hours. Each bottle contained a strip of four indicator-agar filled vials. The absorbance ($OD_{450-590}$) of the agar was immediately read after the CO_2 was measured. The time 0 absorbance values were subtracted from the later measurements and the result was plotted against the volumetric CO_2 concentration. The exponential relationship between % CO_2 and absorbance was well correlated and further used for calculations (r^2=0.83).

4.2.10 Data analysis

All applied C-sources were included into data analysis. MicroResp data were normalized for any differences on the detection plate due to manufacturing. The zero time values were subsequently subtracted from all later measurements of respective plate wells and the data converted into % CO_2 values using the relationship revealed by the calibration. Background respiration values obtained from HMN-amended vials were subtracted before mean values of replicates were calculated. With the first components of principal component analysis (PCA) performed on covariance matrices, which explained more than 99 % of variance, multivariate analysis (discriminant analysis) was carried out using the S-plus 2000 package, MathSoft Inc., MA, USA. The significance between the Mahalanobis' generalized distances between data groups was tested with Hotelling's T^2 test and the Monte Carlo permutation test (103). Hotelling's T^2 statistic is a generalization of Student's t statistic to the multidimensional case (103). No Bonferroni adjustement for multiple statistical tests was applied due to reasons explained in (115). The measurement values of the inhibition experiment were normalized by the background respiration for better comparison between relative activities between soils (Figure 4.4).

4.3 Results

4.3.1 Effect of pre-exposure to kerosene on soil DNA and activity

DNA contents were determined as an indicator for soil biomass. The agricultural surface soil from Aberdeen, Scotland (CWR) contained at the start of the experiment 40.1 µg DNA g^{-1} soil, whereas only 2.1 µg DNA g^{-1} soil was measured in the contaminated subsurface soil from Belgium (AS). The DNA content in both soils was significantly influenced by pre-exposure to the kerosene vapors.

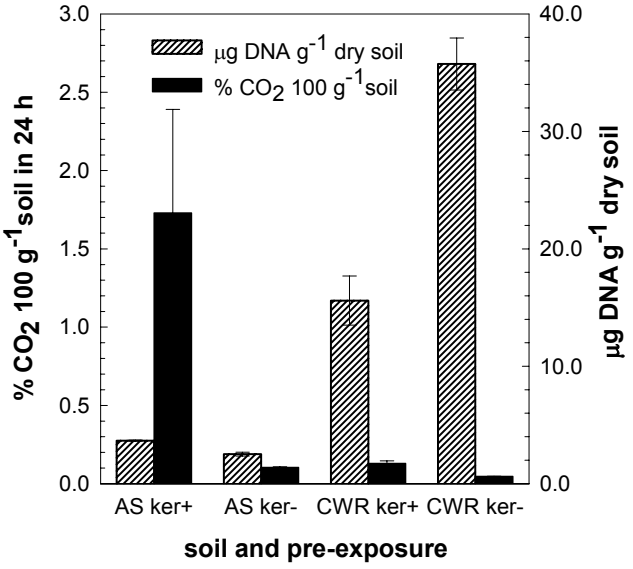

Figure 4.2: Absolute amounts of DNA compared to CO_2 concentrations in incubation jars produced by microorganisms of 100 g soil within 24 hours.

In Figure 4.2 absolute amounts of DNA measured on day 37 are compared with the CO_2 production of 24 hours measured at the same day. The DNA content in the soil AS ker+ increased during incubation to 160 % and in AS ker- to 114 %. In the agricultural CWR soil, the DNA content decreased due to pre-exposure treatments. Only between 31 and 49 % of the initial amount was measured in the CWR ker+ samples, whereas between 71 und 96 % persisted in CWR ker- jars. The four groups AS ker+, AS ker-, CWR ker+, and CWR ker- were all significantly ($p<0.006$) distinguished.

No correlation between soil DNA and respiration was found (Figure 4.2). The CO_2 production was generally higher in jars of AS soil than CWR soil. Both soils were more active in presence of kerosene than without. The groups were not significantly distinguished on a $p<0.05$ level.

4.3.2 Community-level physiological profiles (MicroResp activity)

The pre-exposed soils were incubated on modified MicroResp plates in presence of ten single hydrocarbons (Table 4.2). By filling the MicroResp plates with soil we added to each well of AS ker+ 1.8, of AS ker- 1.3 , of CWR ker+ 4.4 and of CWR ker- 10.0 µg DNA (calculated amount).

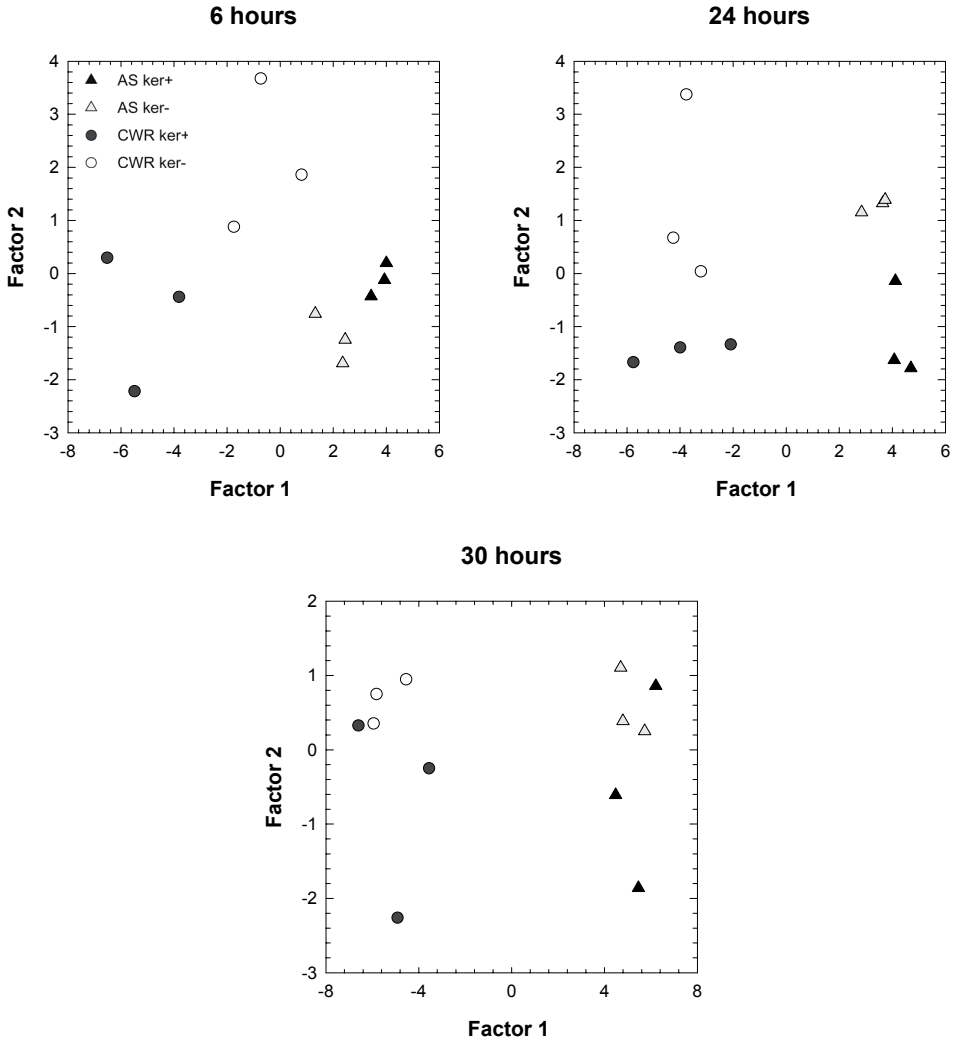

Figure 4.3: Scatterplot diagrams of the discriminant analyses obtained from the Micro-Resp activity experiment show relations between soils and pre-exposure treatments after 6, 24 and 30 hours incubation. Factors explain the following percentages of variation: 6 h: Factor 1 86.2 % and Factor 2 12.4 %, 24 hours: Factor 1 89.2 % and Factor 2 10.5 %, 30 h: Factor 1 98.3 % and Factor 2 1.5 %.

The molar amount of C in the soil water was aimed to be the same in all vials once the equilibrium between organic, gaseous and water phase was established. The low water

solubility of *n*-octane was chosen as basis to be in the non-toxic concentration range for all hydrocarbons. As mentioned earlier, initial concentrations can be calculated with certain constraints. The real initial hydrocarbon concentrations in the soil water lay probably below these theoretical values. With the MicroResp system, we observed negative values in the first measurement period (0-6 hours), documenting reduced respiration in presence of substrates compared to the corresponding background respiration of HMN amended soils. The number of negative values in total and the difference in the overall activity between AS and CWR soil leveled out after 24 hours. The CWR soil was generally more active on the plates than the AS soil. *n*-Dodecane and *n*-decane yielded highest carbon dioxide concentrations in the CWR soil. The AS microbial community had no such clear preferences but showed highest values with aromatics.

The CO_2 values of all substrates used for the MicroResp activity experiment were analyzed with a DA to test the separation power of the modified system between soils and kinds of pre-exposures. Scatterplots of the first two discriminant factors of all three measurement times are shown in Figure 4.3 and significance of Mahalanobis distances between group means are listed in Table 4.3.

Table 4.3: Results of Hotelling's T^2 and Monte Carlo permutation tests of the MicroResp activity experiment for differences between group means based on Mahalanobis distances are shown. Significant values (p< 0.05) are printed in bold.

Groups	6 h			24 h			30 h		
	Hotelling's T^2		Permut	Hotelling's T^2		Permut	Hotelling's T^2		Permut
	F	p	p<0.05[a]	F	p	p<0.05[a]	F	p	p<0.05[a]
AS ker+ - AS ker-	0.30	0.904	-	1.07	0.488	-	0.15	0.975	-
AS ker+ - CWR ker+	4.43	0.197	-	10.23	**0.021**	**+**	10.18	**0.042**	-
AS ker+ - CWR ker-	1.32	0.497	-	10.67	**0.020**	-	11.12	**0.037**	-
AS ker- - CWR ker+	2.91	0.279	-	9.24	**0.026**	**+**	9.72	**0.044**	-
AS ker- - CWR ker-	0.98	0.593	-	7.69	**0.035**	**+**	10.38	**0.041**	**+**
CWR ker+ - CWR ker-	1.66	0.426	-	1.23	0.432	-	0.22	0.946	-

a: + = significant, - = not significant

Neither Hotelling's T^2 nor the Monte Carlo permutation test revealed any significant separation in the data from the measurement 6 hours after incubation start. After 24 hours incubation only the separation on the discriminant factor 1, separating the two soils, was significant (λ=22.36, 89.15 %, p=0.015). This result was reflected in the distances between group means, which were all significant with Hotelling's T^2 test with exception of the ones between the same soils but different pre-exposures. Similar results were obtained with the permutation test. Thus, the method, with the substrate concentrations used in the

MicroResp activity experiment, was able to distinguish the two soils but not the treatments. After 30 hours of incubation the factor 1 (λ=41.08, 98.35 %, p=0.105) is not significant anymore.

4.3.3 Effect-concentration curves with increasing hydrocarbon target concentrations (MicroResp inhibition)

Petroleum hydrocarbons and gasoline additives were applied at different target concentrations to the vials of the MicroResp plates. Figure 4.4 shows the CO_2 production relative to the background respiration of the two measurement periods 0-6 and 6-24 hours. Differences in CO_2 production from different target concentrations could already be observed after 6 hours incubation. The general pattern of low respiration rates at low target concentrations towards higher rates at higher target concentrations and inhibitory effects in the ranges of maximal water solubility for some substances were similar in both measurement periods. The lowest CO_2 production was most often measured at the lowest, and not as may have been expected, at the highest hydrocarbon concentration. Aromatic compounds generally had an inhibitory effect on the microbial activity in their highest concentration. The clearest exception occurred with toluene as substrate for CWR soils. After 24 hours incubation on the plates we detected highest activity at highest toluene concentrations. No clear inhibition of AS soils was observed in presence of m-xylene and 1,2,4-TMB after 6 hours and of m-xylene after 24 hours. *n*-Hexane was easily degraded in both soils without causing any inhibitory effect even at maximal water solubility. The more water soluble *n*-pentane was more toxic: pre-exposed communities showed inhibition after 6 hours incubation at the highest concentration, however this effect disappeared in the second measurement. The most drastic effect in terms of inhibition was observed with ethanol. At pure application, the CO_2 production fell below the background respiration rate after 24 hours incubation.

Differences between the two soils were more pronounced after 6 hours than after 24 hours. The Scottish agricultural soil (CWR) was generally more active than the previously contaminated AS soil, even at high substrate concentrations. An effect of pre-exposure on CWR soil was observed after 6 hours and after 24 hours for all substrates, since the CO_2 production of the ker+ soil was lower. AS soil was much less active and differences between the kerosene exposed and the unexposed samples are hardly visible. This difference was not pronounced in case of the AS soil after 6 hours but became explicit with both *n*-alkanes pentane and hexane after 24 hours.

The CO_2 values at the concentration with the maximum yield (molefraction $x_{i\,mix}$ No 6 for all substrates (Figure 4.4)) were again analyzed with a DA. The scatter plots with the

first two factors are shown in Figure 4.5. This time, using higher target concentrations, we found highly significant distances between all but two groups (Table 4.4) at the 6 and the 24 hours measurement.

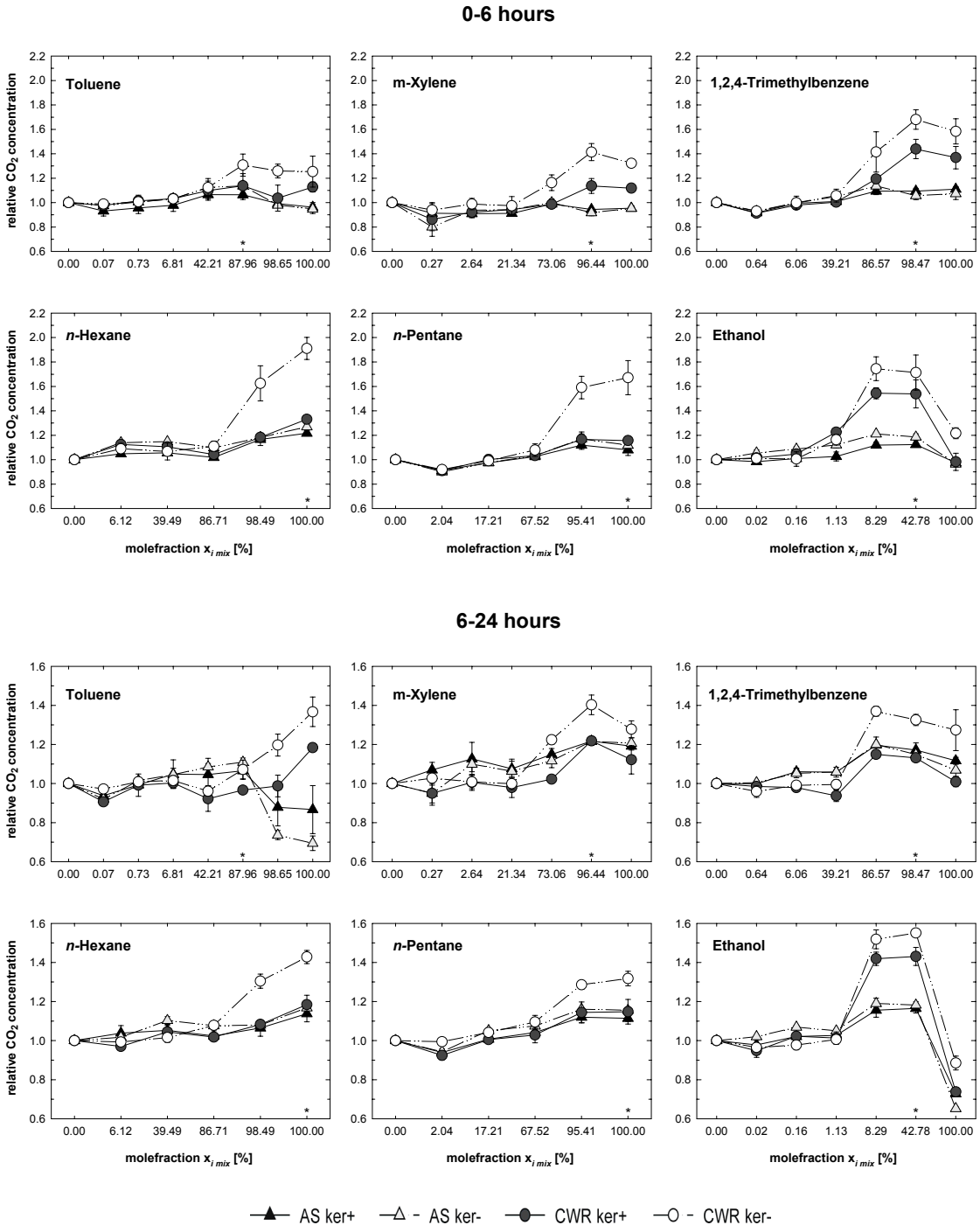

Figure 4.4: Relative CO_2 production shown as function of the molar ratio of the substrate in the HMN/substrate mixture, measured after 6 and 24 h incubation. * Indicates the most CO_2 promoting molefraction (No 6), which was used to perform the DA in Figure 4.5.

These exceptions are the two differently incubated AS soils. The significance levels were generally much higher after 6 hours than after 24 hours. Note that the discriminant factor 1 (λ=99.8, 61.21 %, $p<0.001$) in the left graph (6 hours) separated the only soil which has never been exposed to petroleum hydrocarbons before (CWR ker-) from the ones which have either been contaminated for years and/or have been exposed during the pre-exposure phase of the experiment (AS ker+, AS ker-, CWR ker+). This separation was lost after 24 hours, where factor 1 (λ=72.96, 73.6 %, $p<0.001$) discriminated between the two soils.

Table 4.4: Results of Hotelling's T^2 test for differences between group means based on Mahalanobis distances of the MicroResp inhibition experiment. Significant values ($p<0.05$) are printed bold.

Groups	6 h		24 h	
	F	p	F	p
AS ker+ - AS ker-	2.17	0.280	0.48	0.798
AS ker+ - CWR ker+	10.24	**0.042**	17.47	**0.020**
AS ker+ - CWR ker-	118.35	**0.001**	25.92	**0.011**
AS ker- - CWR ker+	19.59	**0.017**	18.17	**0.019**
AS ker- - CWR ker-	122.92	**0.001**	23.43	**0.013**
CWR ker+ - CWR ker-	102.45	**0.001**	13.66	**0.028**

The lowest target concentrations of the MicroResp inhibition experiment were similar to the concentrations applied on plates in the MicroResp activity experiment. Similar were also the results: After 6 hours incubation, only 2 groups could be distinguished significantly (CWR ker+ - AS ker+), increasing to 4, as in the MicroResp activity experiment, for the second measurement period. The significance levels were lower when high substrate concentrations (No 6) were applied.

4.3.4 Background respiration and heptamethylnonane degradation

CO_2 production in the non-amended and the HMN-amended soil was nearly equal with an average difference of $OD_{450\text{-}590}$ 0.011 or 1.9 % of the measurement value at 24 hours (Figure 4.6). The standard deviation of $OD_{450\text{-}590}$ did not exceed that of non-amended soils at the 24h-measurement (Table 4.5). The standard deviation of the background absorption relative to the measurement values was with 3.3 % higher at the 6 hours measurement than at 24 hours.

The influence of hydrocarbons on the polystyrene detection plates and agar was assessed, since aromatic hydrocarbons have reacted with and deteriorated many materials (see

Chapter 3). The absorbance of the cresol agar detection plate, which was mounted on a bottom plate containing only glass beads and hydrocarbons, was due to atmospheric CO_2. Data show standard deviations, which were very similar to the background respiration, with the exception of the first measurement.

Table 4.5: Background respiration and its standard deviation given as absorbance and the exponentionally related %CO_2, assessed in 72 vials at two different exposure times in two the different soils.

Soil	Hours of incubation	% CO_2	average $OD_{450-590}$	stddev $OD_{450-590}$	CV [%][a]
CWR ker- 2	6 h	0.117±0.003	0.235	0.012	5.30
CWR ker- 2	24 h	0.230±0.010	0.537	0.019	3.53
AS ker+ 2	6 h	0.140±0.005	0.317	0.015	4.60
AS ker+ 2	24 h	0.292±0.010	0.644	0.015	2.33

a: CV=coefficient of variation

4.4 Discussion

4.4.1 Soil DNA and respiration

Pre-exposure to kerosene caused a decrease of DNA content but an increase in CO_2 production in the agricultural soil (CWR), whereas in the previously contaminated soil AS, the CO_2 production and the DNA content increased. The activity, which was measured at the end of pre-exposure, was not correlated to the soil DNA content, a phenomenon which was observed before (32, 133). Caparello and LaRock stated that a large population of less active oxidizers and another smaller, but more active population could produce similar amounts of CO_2 and therefore be interpreted as equal population. Numerically equivalent populations may not be equivalent in their ability to assimilate hydrocarbons. Anderson and Domsch (11) found a good correlation between maximal respiration rates and biomass immediately after glucose addition to the soil. Although we did not assess maximal respiration rates, since CO_2 would have needed to be measured hourly, we found indeed also a better correlation between the amount of DNA and respiration looking at CO_2 productions on the MicroResp system in the 0-6 h measurement period in the non-toxic range of substrates of the MicroResp inhibition experiment. The ranking of CO_2 production rates (CWR ker- > CWR ker+ > AS ker+ > AS ker-) corresponded to the DNA content (Figure 4.2). The magnitude of the substrate induced respiration response within the first 6 hours is characteristic of the initial community in soil, able to degrade the substrate, before growth of organisms occurs on the added substrates (11). The biomass-correlated respiration seemed to disappear already after 24 hours. Hence, activities did not change

proportionally, which may have had different reasons such as limiting nutrients (94) or inhibiting effects (Figure 4.4, 1,2,4-trimethylbenzene). In the MicroResp inhibition experiment, the target concentration of 0.466 mM 1,2,4- TMB (molefraction 98.5 %) induced maximal respiration within the first 6 hours, but was inhibitory in the second 18 hours.

According to the literature (105, 126), an increase in soil biomass was found in the majority of hydrocarbon contaminated soils. The decrease in the DNA content in the CWR ker+ soil seems therefore unusual at the first sight. Oxygen or hydrocarbon depletion were excluded as explanation for this effect since bottles were regularly opened and hydrocarbon sources exchanged. Nutrients are unlikely to be a limiting factor in this soil. But it is probable that the artificial hydrocarbon mixture had toxic effects on a sensitive part of the microbial community, although none of the compounds was present in high concentrations. DNA in surface soils could also originate from other sources than bacteria such as plant debris, fungi, algae, and protozoa. Biomass quantification via the DNA was used by Marstorp *et al.* (108). Taylor *et al.* (147) compared their method to a variety of other methods to assess the microbial activity or to estimate biomass. Harris (75) and Taylor *et al.* (147) considered the contribution of eukaryotic sources to total DNA negligible, specially since extraction of fungal DNA requires harsher extraction methods than bacterial DNA.

4.4.2 Community-level physiological profile (CLPP) of hydrocarbons

Biolog^{TM} plates were recently used as simple method to assess community changes in soils due to environmental changes, among others due to petroleum contamination (17, 28, 49). Biolog^{TM} plates do not provide specific substrates related to hydrocarbon contamination. The use of more pollutant-specific substrates is supposed to provide more detailed information in terms of adaptation of the community to the contamination. In the MicroResp activity experiment, soils were incubated with hydrocarbons at low target concentrations, since toxic effects should be avoided. Various hydrocarbon concentrations were applied to samples in the MicroResp inhibitory experiment. CLPPs were assessed for target hydrocarbon concentrations provoking lowest (No 2, results not shown) and highest (No 6) microbial activity. Comparing results of the CLPPs, we conclude that it was the first measurement period of relatively high substrate concentrations that best reflected the expected pattern from our soil sample. This finding confirms the short measurement duration used with MicroResp in (30) with readily water-soluble substrates to be the most appropriate.

A CLPP based distinction between soils is obtained only, if the variation is small within equally treated soil samples, but large between different soils or treatments. Each soil has

a basal respiration, which is detectable with the MicroResp system. The effect of the substrate mineralization has to be higher than the variation of the background respiration. The detection limit in analytical chemistry is usually determined by three times the standard deviation of the background measurements (95). The absorption of background respiration of soils is given in Table 4.5. The relationship between $OD_{450\text{-}590}$ and % CO_2 is exponential, therefore the absorption values in this paragraph were not transferred to % CO_2 for better comparison between different absorption levels. CWR ker- 2 at 24 h produces the highest standard deviation ($OD_{450\text{-}590}$ 0.019), the detection limit being $OD_{450\text{-}590}$ 0.057 according. Only the 4 easily degradable substrates *n*-decane, *n*-dodecane, toluene, 1,2,4-TMB were inducing a respiration exceeding those values after six hours incubation. After 24 hours all substrates with the exception of *n*-hexane, MTBE and isooctane promoted respiration rates higher than the detection limit. The separation power thus increased. At the concentration level No 6 (maximum CO_2 release for all six hydrocarbons) of the MicroResp inhibition experiment, the detection limit was exceeded for all hydrocarbons in both measurement periods.

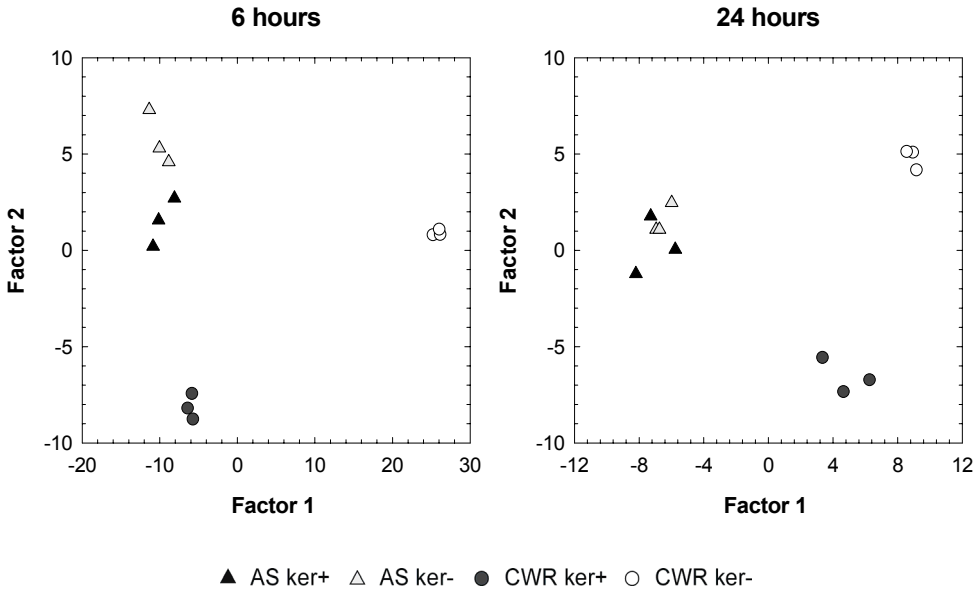

Figure 4.5: Scatter diagrams of the discriminant analyses of initial target substrate concentrations yielding highest CO_2 values (No 6) in the MicroResp inhibition experiment are plotted for both measurement periods. Factor 1 (89.5 %, λ=336.5, p<0.001) and 2 (10.2 %, λ: 38.22, p=0.003) of the 6 hour measurement are represented in the left graph, factor 1 (73.6 %, λ=72.96, p<0.001) and 2 (25.8 %, λ: 25.6, p=0.012) of the 24 hours values in the right one.

These values were used for a second CLPP. Comparing DA outputs of MicroResp activity and MicroResp inhibition (molefraction No 6, see Figure 4.4) it becomes evident that

much more significant results were obtained with higher concentrations. Further do these results correspond to the expectations that pre-exposed samples are separated from the single completely pristine one (CWR ker+). Only factor 1 of the 0-6 hours measurement using molefraction No 6 separated the never contaminated CWR ker- soil from the contaminated ones (CWR ker+, AS ker-, AS ker+) (Figure 4.5). In the second measurement period factor 1 separated the two soils, whereas factor 2 distinguished the treatments, suggesting a decreasing impact of pre-exposure. The reason may be growth, which covers the treatment effect. It has been shown before that communities can adapt very fast to hydrocarbon addition (79, 123). Using results of lower concentrations (either from MicroResp inhibition experiment, molefraction No 1 (scatterplots not shown), or from MicroResp activity experiment) soils were significantly distinguished but treatments could not be separated, neither in the first nor in the second measurement period.

4.4.3 Effect-concentration curves

The MicroResp system is a tool to study respiration of organisms that are not extracted from their natural environment. Degradation as well as toxicity is not a substance property only, but it is the combination of the substance, the organisms, the conditions, and the exposure duration that can cause effects, be they stimulating or inhibiting. The effect of hydrocarbons applied in different concentrations was measured during two periods: from 0-6 hours and 6-24 hours. As mentioned earlier, the first period includes initial respiration peaks as have been observed in (11, 44). The second period includes respiration for cell maintenance but also CO_2 production due to microbial growth. Hydrocarbons for the effect-concentration curves were chosen according to their known toxicity for eukaryotes or eukaryotes and prokaryotes. For *n*-pentane and *n*-hexane little information about environmental toxicity or toxicity towards bacteria were found, since they are biodegradable and volatile, and disappear from soils within hours or days (158). Nevertheless, Garnier *et al.* (63) detected inhibiting effects of concentrations as low as 85 $\mu g\ l^{-1}$ or 0.001 mM *n*-pentane during co-metabolic biodegradation of MTBE by *Pseudomonas aeruginosa* grown on *n*-pentane. Hornick *et al.* (82) suppose that C_2 to C_6 alkanes are inhibitory because they penetrate the membranes. In our study we observed an inhibition of soil microorganisms at lowest *n*-pentane target concentration (target concentration 0.008 mM, molefraction 2.04 %) only in the first six hours. *n*-Hexane never induced toxic effects. With pure ethanol as substrate we observed the clearest results in terms of inhibition. The applied ethanol partitioned to 95 % (v/v) into the soil water at the highest target concentration. In the second measurement period the respiration fell below the background CO_2 production at this concentration. This result was expected since ethanol is a disinfectant and confirms that the method is working. The chosen aromatic compounds showed toxic effects in preliminary experiments or are known to be toxic from literature (43, 86). Tol-

uene had a stronger toxic effect on the AS soil than on the agricultural CWR soil. This difference in its effect was highest at pure application of toluene. This result is rather astonishing, since the AS soil has been BTEX contaminated and AS ker+ and CWR ker+ were exposed to toluene during pre-exposure. Toluene is known to have a toxic effect at already a tenth or a hundredth of its maximal water-solubility on many bacteria (25) or bacterial consortia. Only few strains are known to survive in an environment saturated with pure toluene (86).

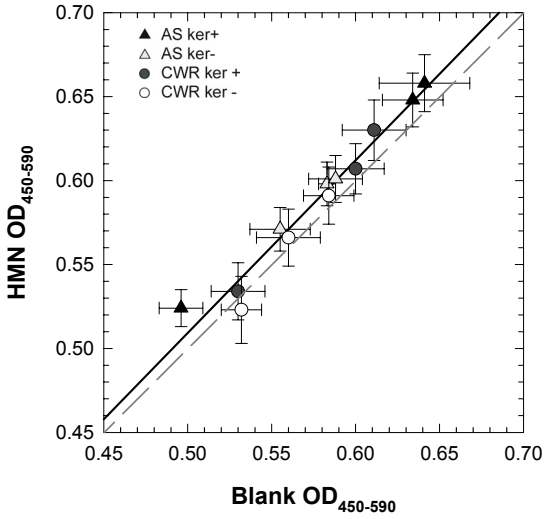

Figure 4.6: Response ($OD_{450-590}$) of detection plate over soil only (Blank) compared to HMN amended soils of the MicroResp activity experiment. The solid line is the correlation, the broken line indicates the 1:1 ratio. Values were measured after 24 h.

The most probable explanation for the limited effect on bacteria in CWR soil is that they are not exposed to the same substrate concentrations as AS soil bacteria *e.g.* due to high adsorption of toluene to the soil organic matter, a factor which was not included in our concentration calculations shown in the Appendix I. Schwarzenbach and Westall (139) found a highly significant linear relationship between the carbon content of the sorbent, in our case the soil, the mass fraction of a non-polar compound, *e.g.* toluene, adsorbed to the sorbent, and K_{OW}, the octanol-water partitioning constant. Sorption is reversible but may have an effect on transport in the soil (139). Indeed, the carbon (organic matter) content in the CWR soil was more than five times higher than in the subsurface soil AS and may have been the reason for toluene not being toxic in CWR soils. Although the affinity of m-xylene and 1,2,4-TMB to soil organic matter is higher than that of toluene (log K_{OW}: toluene 2.73, m-xylene 3.20, 1,2,4-TMB 3.78 (74)), and hence the adsorption is bigger, both compounds show inhibiting effects. Toxic concentrations (LC_{50} or EC_{50}) found in

literature (25) are in general lower for m-xylene and 1,2,4-TMB than for toluene, although no study for microorganisms was found, which compares all three compounds in exactly the same way. Toluene toxicity is due to the interaction of the compound with the cytoplasmic membrane by loss of the cations Mg^{2+} and Ca^{2+} as well as other small molecules (43). The inhibition test applied in the present study can potentially be used as a test to determine quality values that aim protection of soils or sediments, if applied with a higher resolution in the high concentration ranges (159).

We observe in the second measurement period at lower concentration ranges of all aromatics and *n*-hexane a higher activity in AS soils than in CWR soils. This might show the adaptation of the earlier contaminated soil microorganisms to low pollution concentrations, which the AS soil bacteria are able to use as a substrate, in contrast to CWR bacteria, which are used to a C-rich environment. A phenomenon to be discussed is the respiration decrease, compared to the 0 level, at the lowest concentration being applied. This effect is pronounced for m-xylene, toluene, but also *n*-hexane and *n*-pentane after 24 hours incubation. The same effect was observed also in the MicroResp activity experiment. We suppose that the presence of these hydrocarbons already have inhibiting effects but are not yet concentrated enough to serve as a valuable substrate for many bacteria. In the organic matter-poor AS soil we measure more negative values than in CWR soil, probably caused by the lower organic matter content in the AS soil, as discussed above.

With this study we were able to demonstrate that hydrocarbon exposure to kerosene affected both DNA amount and soil microbial activity. The modified MicroResp system proves to be a useful tool to assess specific CLPPs based on biodegradation of VOCs. However, only relatively high, but for the majority of strains non-toxic, hydrocarbon concentrations produce a highly significant pattern. The optimal target concentrations for a CLPP can be found by applying a range of increasing target concentrations as demonstrated in the MicroResp inhibition experiment. In both, the MicroResp inhibition and activity experiment the carrier phase HMN allowed to maintain almost constant concentrations during incubation periods up to at least 30 hours.

4.5 Acknowledgements

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CHAPTER 5

Rapid testing of volatile petroleum hydrocarbon degradation in nutrient amended soils using a micro-respiration system

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submitted to Environmental Microbiology

5.1 Introduction

Engineered bioremediation of petroleum-contaminated soils consists of providing favorable conditions of nutrients, oxygen, temperature, and moisture content to maximize biological hydrocarbon degradation rates. The identification of rate-limiting factors is thus a prerequisite for any successful bioremediation and has been done traditionally in time-consuming and costly laboratory batch and microcosm experiments (153). Nitrogen and phosphorous are the nutrients most frequently limiting microbial activity in organically polluted soils (112). Zhou and Crawford (175) showed that the optimal ratios may be different for different soils, and Thornton-Manning *et al.* (153) stated that nutrient requirements even within one soil are dependent on the soil horizon. They found that nitrogen

and phosphorous did not stimulate phenol mineralization in the upper horizons of two soils in Alabama, but did stimulate phenol mineralization in the subsurface soil.

The quantity of nitrogen and phosphorous required to convert all of the petroleum carbon to biomass may be calculated from the carbon to nitrogen (C:N) and carbon to phosphorous (C:P) ratios found in cellular material (47). Accepted values for a mixed microbial population in the soil are a C:N of 10:1 (161) and a C:P of 100:1 (152). In reality a complete assimilation of petroleum carbon into biomass is never achieved under natural conditions. One reason is the biochemical recalcitrance of some petroleum hydrocarbons. The degree of conversion of readily metabolised substrate is characterized by the efficiency or yield coefficient accounting for the loss of carbon due to mineralization to carbon dioxide. Moreover, not all the substrates and nutrients in soil may be bioavailable due to sorption and precipitation processes. Hence the optimal C:N and C:P soil ratios are expected to differ from theoretical values. Numerous studies have addressed the problem of amount and form of nutrient amendments to achieve hydrocarbon degradation. Zhou and Crawford (175) evaluated the kinetics of BTEX (benzene, toluene, ethylbenzene, xylenes) degradation as a function of nutrient application. They showed that different forms of N (NH_3 as vapor, NH_4NO_3) were equally effective as an N source for BTEX degradation, but found a shorter lag phase with ammonia. Mills and Frankenberger (114) have shown that the addition of K_2HPO_4 at up to 500 mg kg^{-1} enhanced degradation of diesel fuel.

The objective of this study was to develop an easy, miniaturized and rapid method to test combinations of environmental factors on petroleum hydrocarbon degradation without using the classical microcosm approach (153). Here we report on the influences of N and P additions on the biodegradation of toluene, using a modified form of the MicroResp system (30) optimized for the use of volatile, potentially toxic substrates.

5.2 Materials and methods

For the experiments we used a hydrocarbon-contaminated sandy soil from a site below a commercial petrol station in Assebroek, Belgium, which had been operating for 10 years (Table 5.1). On the site, BTEX and MTBE (methyl *tert*-butyl ether) concentrations between 380-1000 and 300-5400 μg kg^{-1}, respectively were previously measured in borehole samples (Gunther De Becker, pers. comm.). The fine soil was sieved (<2mm) and stored at 25°C. Total Ca^{2+}, Mg^{2+} and K^+ were analyzed before the experiments began. To extract the analytes, 0.43 M acetic acid was mixed with the sieved, dried and ball-milled soil (soil:extractant ratio of 1:40) by means of an end-over-end shaker. The suspension was filtered through a Whatman No 542 filter and the filtrate subsequently analyzed by inductively coupled plasma – atomic emission spectroscopy (ICP-AES).

Extractable N was determined after extraction of air-dried soil with 1 M KCl for 60 min (soil:extractant ratio of 1:5) and filtration through a Whatman No 40 filter. P was extracted with distilled water (soil:extractant ratio 1:10) and filtered first through a Whatman No 42 filter, then through a 0.45 μm filter. NH_4-N, NO_3-N and PO_4-P were analyzed colorimetrically in the resulting filtrate using a flow injection analyzer (Perstorp Application Note AN65/84, ASN 65-32/84 for ammonium; AN65/84, ASN 65-31/84, 62/83 for nitrate; ASN 60-01/83 for phosphate). Total carbon and total organic carbon (TOC) were determined using a Carlo-Erba elemental analyzer.

Table 5.1: Selected soil properties of the unamended Assebroek soil. Concentrations are given in [mg kg^{-1}] unless indicated differently.

Source	Measurement value
pH	7.8
Initial gravimetric water content [g g^{-1} wet soil]	0.048
Water holding capacity (WHC)[%]	14
Total carbon [g kg^{-1}]	8.3
Total organic carbon [g kg^{-1}]	6.3
Extractable P	5.2
Extractable NH_4^+	1.21
Extractable NO_3^-	2.77
Acid-extractable Mg^{2+}	143.6
Acid-extractable Ca^{2+}	6880
Acid-extractable K^+	96.0

Three lots of 80 g soil with an adjusted water holding capacity (WHC) of 14 % were stored for 25 days in 1.5 l bottling jars (Kilner®, Ravenhead Company Ltd., Staines, UK) at 25°C until they were filled into the MicroResp system (30). Briefly, this system consists of a CO_2-detection microtiter plate placed upside down on a soil- and substrate-containing deepwell bottom plate. The system is tightly held together with a gasket. Corresponding wells are connected by holes in this gasket to allow CO_2 to diffuse from the bottom into the detection plate. Since petroleum compounds can deteriorate styrene-based plastics, a modified MicroResp system (Figure 5.1) which used glass for the bottom reaction plate (Multi-Tier™, Wheaton Scientific Products Ltd., Milleville, NJ, USA) was applied. This plate consists of a base plate with 96 removable glass vials (Figure 4.1 a). These were sorted by length (± 0.1 mm) so as to present an even surface to the gasket. 0.3 g glass beads (∅ 0.25-0.56 mm), previously muffled overnight at 500°C, were filled into each well before the plates were autoclaved and dried. The interstitial space between the glass beads

served as a recipient for the liquid hydrocarbon phase added later. The soil was filled into the 96 glass vials following the method described in (30) using a filling plate, such that each vial contained 0.53 ± 0.040 g moist soil. Nutrient stock solutions with 3.7 M NH_4NO_3 or 1.86 M K_2HPO_4 were diluted with sterile milli-Q water to give the desired concentrations. 85.7 µl was pipetted onto the soil of each vial in order to obtain a final moisture content of 60 % of the WHC. Nutrient concentrations were chosen targeting initial molar C:N ratios of >460:1, 460:1, 92:1, 46:1 and initial C:P ratios of >2130:1, 2130:1, 213:1, 21:1, where the C represents toluene-C. Background N, P and organic C were not considered in the calculations but a control without added N or P was included into the experiments. Each of the 16 permutations resulting from applying four N and four P concentrations were applied to each of the three incubated soil lots in quadruplicate. Thus, a total of 192 vials were used in two 96-well plates.

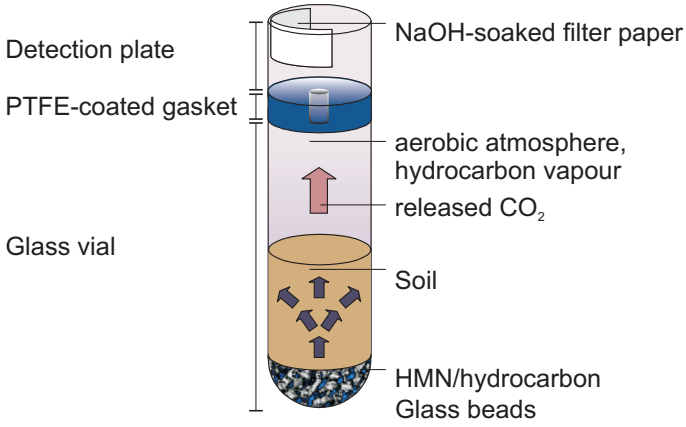

Figure 5.1: Modified MicroResp system: schematic lateral view of a glass vial connected via the gasket to the detection well containing the filter paper.

Filling of the plates and adding the nutrient solutions stimulated microbial activity (data not shown) and hence immediate measurements after filling might mask the activity due to increased nutrient availability. Further, precipitation and sorption of nutrients were assumed to take place in the first few hours after their addition. Therefore the prepared plates were stored for 72 hours in air-tight boxes containing soda lime as a CO_2 trap and a wet tissue to avoid desiccation. ^{14}C-UL-toluene and 2,2,4,4,6,8,8-heptamethylnonane (HMN, purity 98 %, both purchased from Sigma-Aldrich Company Ltd., Gillingham, UK) were mixed with non-radiolabeled toluene. HMN is a biochemically very inert compound (73) with low vapor pressure. It serves as a matrix for controlling the vapor phase and delivery of the toluene without building up toxic concentrations. Thirty µl toluene-HMN mixture was added to each well by means of a syringe needle mounted on a shortened pipette tip. Each vial received 1200 Bq and 5.1 mg toluene. Toluene vapors migrate

rapidly through sandy soils (79, 123), and within the vials an equilibrium toluene vapor concentration governed by Raoult's law (138) establishes

$$C_{i\ (air)} = x_{i\ mix} \cdot \gamma_{i\ mix} \cdot C_i^{sat} \qquad \text{(eq. 5.1)}$$

$C_{i\ (air)}$ being the actual toluene vapor concentration in the vial before biodegradation starts, $x_{i\ mix}$ the mole fraction of toluene in the organic mixture (1:5.09), $\gamma_{i\ mix}$ the activity coefficient (1 for hydrocarbons) and C_i^{sat} the vapor concentration over pure toluene (138 g m^{-3} air at 25°C). The resulting theoretical aqueous concentration of toluene in the soil water, calculated with Henry's law, is 2.3 mM, a concentration at which highest growth rates are observed (37). Hydrocarbon loss was minimized by sealing the vials with a lid immediately after application of the mixture. A small filter paper (10 mm × 5 mm) soaked with 20 µl 4 M NaOH was placed along the wall of each well of the styrene detection plates to trap the $^{14}CO_2$. Once the substrate was added to the whole plate a detection plate was placed upside down on the bottom plate using a modified PTFE layered Web-Seal™ gasket (Chromacol Ltd., Herts, UK) to connect the corresponding wells and to seal the system. The sandwich-type stack of the bottom plate, the seal gasket and the detection plate were additionally pressed together with a clamp and was incubated at 25°C. Two detection plates were used for each deep-well plate, the first for the incubation time of 0-24 hours and the second for 24-48 hours. After removal of the top-plate, 200 µl of scintillant (Wallac Optiphase Supermix) was added to the detection-wells, completely covering the filter papers. The radioactivity was determined with a microplate liquid scintillation counter (Wallac MicroBeta TriLux counter, Wallac Oy, Turku, Finland). Toluene-HMN mixture applied to autoclaved soil served as blank to determine the amount of labeled toluene accumulating in the filter paper. This amount was less than 0.1 % of the toluene-C after 24 hours, also confirming theoretical estimates, and was subtracted from the values obtained from experimental soils. The activity of 30 µl of HMN-toluene mixture was determined in order to calculate the counting efficiency (measured activity divided by the theoretical activity), this being 85.6 %. Activity values were corrected with this counting efficiency before a two-way ANOVA using Genstat 6, release 6.1 (VSN International Ltd., Oxford, UK) was carried out. The samples were grouped according to the nutrient type and their concentration. A Monod-type curve, which takes into account the inhibition effects of high nutrient concentrations,

$$\mu = \frac{\mu_{\max} S}{K_S + S + \frac{S^2}{K_I}} \qquad \text{(eq. 5.2)}$$

was fitted to the mean values from statistically significant treatments using KaleidaGraph (release 3.5, Synergy Software Technologies Inc., Vermont, USA). In (eq. 5.2), μ is the specific growth rate expressed as $^{14}CO_2$ production in our experiment, S is the added nutrient concentration, K_S and K_I are saturation and inhibition constants, respectively. The optimal nutrient concentration was calculated by the derivation of (eq. 5.2)

$$S_{opt} = (K_S K_I)^{0.5} \qquad \text{(eq. 5.3)}$$

5.3 Results

Figure 5.2 shows that within 24 hours, a maximum of 0.73 % of the added ^{14}C-toluene was found as $^{14}CO_2$, whereas from 24 to 48 hours, the maximum recovery was 2.84 %. Controls including vials with toluene-HMN mixture only or vials with toluene-HMN and autoclaved soil yielded maximum recoveries of radioactivity on filters used as CO_2-trap of 0.08 %.

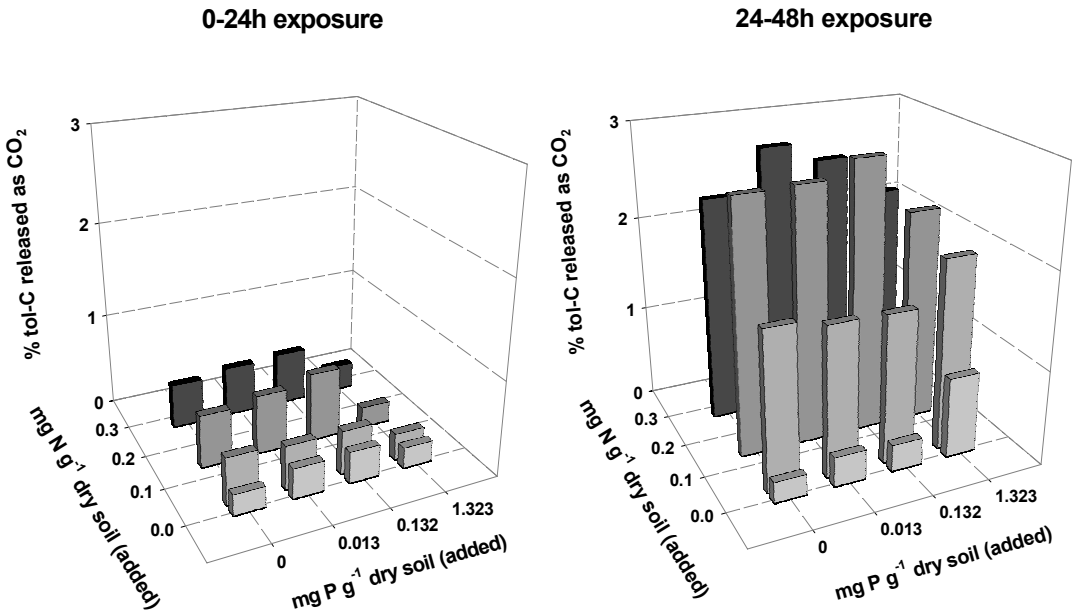

Figure 5.2: Mean measured % toluene-C released as CO_2 between the first 24 hours (left graph) and between the second 24 hours of the overall 48 hours measurement period (right graph).

The three soil lots behave very similarly with the coefficient of variation (CV) being on average 5.7 %, exceeding rarely 10 % (Table 5.2). These CVs are only slightly bigger than within four replicate vials from one lot, where a mean CV of 5.06 % was found.

The addition of nutrients had a stimulating effect on the toluene degradation. A generally higher mineralization rate was observed in both N and P amended soil in the second measurement phase compared to the first one. However the CO_2-production did not increase in the blank and the wells to which only low phosphorus concentrations were added (Figure 5.2). The two-way ANOVA of the 16 permutations of 4 N and 4 P concentrations (Table 5.2) showed an overall significant influence of N-addition on toluene degradation in both measurement periods, whereas the effect of phosphorus was only significant in the first 24 hours. Nevertheless, between 24 and 48 hours, addition of P alone resulted in an increased but low mineralization activity only for the highest P-concentration. If P was added in combination with N, optimum P concentrations were at intermediate levels (Figure 5.2). That is, the requirement for P for optimal toluene degradation was partly offset by the level of N (Figure 5.2). The maximum mineralization in our samples at both measurement periods occurred at 0.137 mg added N g^{-1} dry soil and 0.132 mg added P g^{-1} dry soil, corresponding to a final C:N:P ratio of 213:2.3:1.

Table 5.2: F values, significance (p values) and coefficient of variation (CV) of the two-way ANOVA.

	24 h			48 h		
Treatment	F	p	CV*	F	p	CV*
Nitrogen	29.45	<0.001	5.13	888.59	<0.001	3.21
Phosphorus	35.70	<0.001	5.24	2.47	0.080	1.87
Nitrogen + Phosphorus	4.11	0.001	11.20	18.46	<0.001	7.67

* coefficient of variation, being the percentage of the standard error of the mean of each treatment

Although we used only four concentrations for each nutrient, a Monod-type curve (eq. 5.2) was fitted to mean $^{14}CO_2$ production rates of samples which were only N-amended. Figure 5.3 shows the fit (r^2=0.94) for the second measurement period. The values of the unknowns are μ_{max}=3.58 [% of toluene C released as CO_2 within 48 hours], for K_S=0.03 [mg g^{-1}], and K_I=0.83 [mg g^{-1}] so that the highest microbial activity would be expected at a N concentration of 0.157 mg N g^{-1} soil, calculated from (eq. 5.3). Hence the optimal N concentration is found to be between the two highest N concentrations applied suggesting an optimal C:N ratio of 69:1. The degrees of freedom were restrictive for significance testing of these fitted functions with this low number of samples. However, our calculations illustrate how results obtained with our modified MicroResp system can be utilized to determine optimum nutrient amendment.

5.4 Discussion

The modified MicroResp system is a valuable tool for the rapid screening of rate-limiting nutrients for hydrocarbon degradation in soil. Two modifications were needed with respect to the conventional MicroResp system for applying of volatile toluene. Firstly, glass vial inserts had to be used instead of disposable plastic microtiter plates because toluene deteriorates polystyrene and polypropylene. Secondly, the toluene had to be added as an HMN-mixture to the bottom of the vials below the soil instead of being added as an aqueous solution to the soil (Figure 5.1). This serves to supply toluene to the soil microorganisms at non-toxic concentrations via the vapor phase. The HMN ensures a constant toluene vapor concentration over extended time. Although not tested in this study, the system could be used for longer incubation times, however attention has to be paid on O_2 depletion. Confidence in the method is gained from the high reproducibility within replicates and between soil lots in spite of the relatively small soil sample size (Figure 5.1), suggesting also that our soil samples were homogeneously mixed. Ellingsøe and Johnsen (54) compared microbial community variability within different sizes in a forest soil by DGGE and concluded that variation in samples with sizes $\geq$1 g are negligible, but occurred in samples $\leq$0.1 g. The variation will also depend on the soil texture being potentially greater in an organic than in a sandy soil. We generally used 0.53 g soil per vial, but we assume that the variability on the physiological level is lower than on the genetic one, hence loss of biochemical diversity may be less likely than of genetic diversity.

The increase in toluene degradation in the amended wells shows a nutrient deficiency in the soil. At the C:N:P ratio giving the highest toluene degradation, the rate was increased 3.3 and 13 times over the control during the first and the second 24 h of incubation, respectively. However, highest nutrient concentrations were found to have negative effects. This shows that determining the exact N and P requirements is not only economically important but also importantly avoids overdosing with deleterious effects on both the bioremediation process and the environment. Zhou and Crawford (175) observed the same phenomenon at a C:N ratio of 1.8:1 and assumed that ammonium toxicity inhibited microbial growth. This should not be the case with a pH of 7.8 in our soil. The explanation we suggest for our experiments is that excessive nutrient addition leads to growth-inhibition due to high osmotic pressure, which was previously described by Mills and Frankenberger (114). Wells with C:N/C:P of 46:1/21:1 had a theoretical final concentration of 31.4 g l^{-1} K_2HPO_4 and 2.9 g l^{-1} NH_4NO_3 in the soil water without considering adsorption. Assuming that none of the ions was adsorbed onto the soil matrix, these concentrations would correspond to 10 times average growth medium concentrations for non-halophile bacteria. The idea that osmotic pressure could have a negative effect on some strains is supported by the fact, observed in the second measurement period (Figure 5.2), that the

more N was added to the well, the lower was the P concentration leading to the maximal CO_2 yield. An alternative explanation of the lower mineralization efficiency at high nutrient levels would be the possibility of a shift in microbial metabolism (with greater C assimilation efficiency at the higher nutrient concentrations) and/or a shift in the microbial community responsible for degradation.

This obvious interaction between the added amount of N and P was not included in the curve fitting, neither were the background levels of nutrients. Considering the background levels would in our case have meant fitting a function of four unknowns through four sample points, *i. e. S* in (eq. 5.2) would have needed to be substituted by *S*+*d* with *d* representing the background N concentration. This is leading to a perfect fit due to a degree of freedom of 0. By doing so a background N concentration of 3.8 mg kg^{-1} soil was estimated, a value similar to the measured extractable N, which was 1.6 mg kg^{-1} soil. By including more concentrations of N and P in the experimental design, which is easily possible with the MicroResp system, bioavailable background nutrient levels can be modeled and significance tests incorporated in the data analysis.

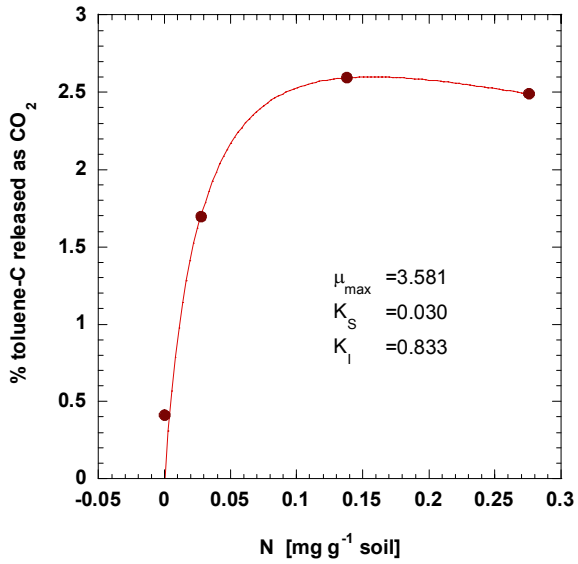

Figure 5.3: Measured % toluene-C released as CO_2 from 24-48 hours for only N amendet wells (symbols) and best fit of Monod-curve (eq. 5.2).

While we tested N and P limitation, this modified MicroResp system is potentially useful for a number of other applications as *e.g.* the optimization of soil pH, moisture content, the testing for limiting micronutrients, or conversely testing the toxicity of substrates and elements. The system is potentially also applicable for incubations under anaerobic conditions, which can be relevant for deep soil surfaces and sediments. Thomas *et al.* (151)

state that biodegradation in the subsurface will be site-specific and even microsite-specific and hence samples from several cores and depths should be tested to get realistic rate data that are applicable to the scale of a contaminated site. We believe that our simple and rapid method will allow the experimenter to handle many more samples than conventional microcosm methods.

5.5 Acknowledgements

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CHAPTER 6

General Discussion and Outlook

In this thesis, biodegradation of VOCs in soils, concomitant microbiological community changes and factors limiting the biodegradation were assessed with miniaturized physiological methods. The development of microbial activity and total biomass in presence and absence of petroleum hydrocarbons were determined in sand and soils, in the field and in microcosms. The response of soil microbial communities to hydrocarbon contamination was characterized by 1) estimations of specific hydrocarbon degraders, 2) physiological profiles with two different miniaturized methods, 3) the CO_2 production as a measure for metabolic activity, and 4) total cell counts or DNA concentrations as indicators of total biomass.

The main task of this thesis was to provide a new physiological test that can be used to detect changes in the microbial soil communities as a response to contamination with volatile hydrocarbons. Table 6.1 gives an overview of the methodological approaches used and modified during this work, whereas Table 6.2 lists the observed advantages and disadvantages of these methods. In recent years several methods were applied to investigate microbial community function or the functional diversity in hydrocarbon contaminated soils (17, 24, 28, 49, 126, 172) or to enumerate petroleum-degrading bacteria (12, 27, 38, 90, 113, 171). The classical Biolog™ plates were very popular and most widely applied, because they are simple to use and yield a great deal of information (31). The analysis and interpretation of the data is more complicated and doubts arose whether Biolog™ substrates and their concentrations, which were originally chosen to support growth of bac-

teria of medical relevance, are ecologically meaningful and actually suited to characterize functional diversity in the environment (98).

Biolog™ is a culture-based method if the plates are inoculated with less than 10^8 viable cells ml^{-1}, which are all capable of degrading the same range of substrates (169). Except for very easily degradable ones, this condition is rarely fulfilled in soil extracts. The low coefficient of variation calculated for the Biolog™ plates after 72 h incubation with Værløse soil extracts (Chapter 2) shows that even a growth step may not make up for the low cell numbers extracted from subsurface samples. The importance of bacterial growth for color formation alone indicated that the responses were a reflection of functional potential rather than *in situ* functional ability (62). Moreover, evidence was found that only a limited range of microorganisms from a community influences the Biolog™ pattern (71, 160).

Table 6.1: Overview and chronological listing of the methodological developments made during this thesis

Methods	Substrates	Detection	Chapter
Biolog™ EcoPlates	Water-soluble compounds	Crystalline tetrazolium, spectrophotometry	2/3
Biolog™ MT-Plates	Water-soluble compounds, which don't corrode PS	Crystalline tetrazolium, spectrophotometry	Preliminary experiments
WST-MPN	VOCs, which don't corrode PP	Water-soluble tetrazolium, plate scan	3
Conventional MicroResp	Water-soluble substrates, non-toxic VOCs	Cresol red agar (pH indicator to detect CO_2), spectrophotometry or Scintillation counting of $^{14}CO_2$	Campbell *et al.*, AEM 69, p. 3593
Modified MicroResp (pH indicator)	VOCs	Cresol red agar (pH indicator), spectrophotometry	4
Modified MicroResp (^{14}C)	^{14}C-Toluene	Scintillation counting of $^{14}CO_2$	5
Hybrid MicroResp-MPN	VOCs	Cresol red agar (pH indicator), spectrophotometry	Outlook

Researchers replaced or exchanged substrates on Biolog™ plates to adapt the plate to specific environmental investigations. But only few of them applied single volatile substrates in miniaturized systems like microtiter plates (66, 146). The biggest challenge in the work with VOCs is of course their volatility and sometimes their toxicity. To keep substrate concentrations constant over a relatively long period of time, a high amount of substrate in low non-toxic concentrations has to be added to a growth medium (in an air-tight measurement system). This contradiction could only be solved by separating the high substrate amount from the medium and to find a way to continuously release it at small rates thereby keeping concentrations below toxic levels. This goal was achieved by introducing the hardly biodegradable NAPL HMN as a carrier phase for VOCs in the WST-MPN and

the MicroResp method. By knowing partitioning properties of the VOCs, their amount in the water phase of the experimental system can be controlled by applying formulas given in the Appendix I. Effect-concentration curves obtained in the MicroResp inhibition experiment demonstrate probably best that the two-liquid phase system is working. The absolute concentrations to which microorganisms are actually exposed in their microenvironments may be different from those indicated. Concentrations given in Chapters 3-5 have to be considered with caution, since verifying measurements in the soil water are very difficult to perform. Neither all phases of the system nor biodegradation were included in the calculations. Missing are adsorption to the soil, glass and the gasket. Thus, the term 'target concentration' was introduced, which is the theoretically maximal initial hydrocarbon concentration in the soil water.

The WST-MPN method (Chapter 3), as a technique to estimate cell numbers, is a cultivation-dependent method with all inherent shortcomings of these methods such as the selective growth of few strains biasing the results. A yellow signal indicates only that at least one microbe able to degrade the particular substrate was added with the diluted soil extract to the well. The MPN method uses a mineral medium, which is less concentrated, and a formazan dye that is more sensitive than the one provided on the Biolog^{TM} plates. Applying this method with volatile hydrocarbons as substrates and bacteria extracted from alluvial sand pre-exposed to volatile hydrocarbons, it was shown that microbial numbers increased for most of the hydrocarbons already present during pre-exposure. Although isooctane was shown to be slowly degraded in this alluvial sand (Table 1.1 and (123)), no isooctane-degrading bacteria could be detected with the WST-MPN method, neither in pristine nor in contaminated sand. Boosting the bacteria with glucose as single electron donor did also not induce the wished effect. In conclusion, one can state that our test system combining WST-1 detection, glucose as substrate for community activation, and incubation times of 3.5 hours did not allow being sensitive enough to obtain a signal from small populations such as those degrading the branched hydrocarbons. However, the method is by all means able to estimate cell numbers of toluene, MCH or *n*-alkane degraders. To use PP plates instead of glass plates for the MPN method was due to the lack of appropriate glass plates, which met the aim of the project to provide a cost-effective method. However, a less gas-permeable but still chemically resistant material such as glass appeared to be required for using the MicroResp CO_2 detection system. A kit-like solution was found, composed of a base-plate with removable vials, which proved to be very convenient for the filling, subsequent soil arrangement on plates and washing.

Table 6.2: Advantages and disadvantages of miniaturized methods explored in this thesis (with exception of the classical MicroResp system)

Methods	Advantages	Disadvantages
Biolog™ EcoPlates	• Commercially available • Easy to handle	• Developed for medical purposes: High nutrient concentration • Substrates unrelated to environmental questions • Inoculation with soil extracts • Cultivation method • Not sensitive enough for subsurface soil extracts
Biolog™ MT-Plates	• Commercially available • Easy to handle • Substrates can be chosen to address specific questions	• Developed for medical purposes: high nutrient concentration • Inoculation with soil extracts • Cultivation method • Not sensitive enough for subsurface soil extracts • Corrosive plate material (PS) • Unsuitable for volatile substrates
WST-MPN	• Sensitive tetrazolium • Medium adapted to environmental questions • PP plates are more resistant to chemical deterioration than PS plates • Gives number estimates of specific hydrocarbon degrader	• WST-1 is not entirely reliable • PP plates are not gas-tight • Inoculation with soil extracts • Cultivation method if not enough bacteria inoculated • No spectrophotometric measurements possible • Quite expensive
Conventional MicroResp	• Whole soil samples • Cultivation independent • Substrates can be chosen to address specific questions • Sensitive • Gas-tight • Cost-effective (if cresol red detection plates are used)	• Corrosive PS plates • Unsuitable for toxic VOCs • Expensive with radiolabeled substrates
Modified MicroResp (pH Indicator)	• Inert glass plates • Gas-tight • For VOCs and other substrates • Whole soil samples • Cultivation independent • Sensitive • Cost-effective (if cresol red detection plate is used)	• Silicon gasket resistance to VOCs unknown
Modified MicroResp (^{14}C)	• Glass plates • For VOCs and other substrates • Whole soil samples • Cultivation independent • Sensitive • Gas-tight • Origin of CO_2 is traceable	• Silicon gasket resistance to VOCs unknown • Expensive • Limited availability of ^{14}C-labeled substrates
Hybrid MicroResp-MPN	• Inert glass plates • Gas tight • Suitable for VOCs • Reliable detection method with pH-indicator • Less expensive than detection with WST-1 • Number of specific hydrocarbon degrader (estimation)	• Silicon gasket resistance to VOCs unknown

The two-liquid phase system was applied to the modified MicroResp system, using the same hydrocarbons as with the MPN method (Chapter 4). The two major differences between the MicroResp and the MPN method are the cultivation independency of MicroResp and the CO_2 production as indicator instead of the dehydrogenase activity. The method is sensitive: background respiration is easily measured. In Chapter 4 we applied *n*-pentane, *n*-hexane, ethanol, toluene, m-xylene, and 1,2,4-trimethylbenzene in increasing concentrations to pre-exposed and pristine soil samples. Volatile organic carbon concentrations in the range of few µM induced inhibitory effects in case of the three aromatics and *n*-pentane and failed to boost mineralization. Community-level physiological profiles from these concentration levels assessed in the first 6 hours after incubation were unable to distinguish soil type and treatment. By adding VOC concentrations between 0.1 and 6 mM (concentration level No 6 in Chapter 4), depending on the substrate, DA significantly separated soil type and treatment and reflected the pattern we had expected from knowing the contamination history of the soils. An overview of advantages and disadvantages of the newly developed and similar existing methods is given in Table 6.2.

In the experiments described in Chapters 3-5 we have never found a correlation between soil activity and total biomass in incubation flasks. Total biomass, either measured as DNA concentration in the soil or as total cell counts, is therefore a weak parameter to predict bioremediation performance or to draw conclusions on hydrocarbon degradation. Activity and biomass of a soil community exposed to petroleum hydrocarbons are depending on a multitude of factors. At the Værløse field site, cell numbers did hardly increase with increasing petroleum concentration but they decreased with depth, as did the concentrations of nutrients and the organic matter content. With a canonical ordination method, namely redundancy analysis, we were able to show that the four soil parameters total N, total P, pH and the water content explained nearly half of the whole variation within activity and biomass (activity and biomass were grouped together and termed 'biology' in Chapter 2). Only 34 % of variation was explained by the contamination-related factor 'time'. This factor was correlated to CO_2 concentrations but not to total cell numbers. The assumption that total cell numbers and nutrients were very well correlated because the soil was nutrient deficient was confirmed by increasing hydrocarbon degradation hours after ammonium addition (pers. comm. N. Dakhel and P. Höhener). The biomass increase over several orders of magnitude often observed by other authors in hydrocarbon contaminated soil (119, 142) was neither observed in the pre-exposed Værløse nor in the Assebroek subsurface soil (Chapter 4). These soils were nutrient deficient. Sixteen combinations of N and P concentrations were applied to the toluene amended soil and the C:N:P ratio of 213:2.3:1 was found to yield highest toluene degradation. This ratio does not include the soil-inherent nutrients and are therefore lower than the normally accepted values of C:N:P of 100:10:1 (152, 161). Using the MicroResp system with radiolabeled ^{14}C-substrates on

nutrient amended soil samples is an efficient method to find the nutrient composition for optimal degradation activity in a soil (Chapter 5). Fitting results into a Monod-type equation allows calculating the physiologically optimized nutrient concentration combination.

6.1 Unexplored options and outlook

The WST-MPN plates were an attempt to develop a sensitive method to estimate numbers of specific hydrocarbon degraders during a bioremediation project. We tried to apply the method during the GRACOS field experiment and discovered that WST-1 was not a reliable activity indicator. WST-1 is a new product and was not often used for environmental investigations yet. To circumvent the use of the unreliable WST-1 a hybrid between the MicroResp and the MPN method could be imagined. The cresol red agar would be a sensitive and cheaper alternative to detect microbial activity than WST-1, and the glass vial system used in Chapters 4 and 5 could replace the PP plates. Further effort should be made to determine ideal application concentrations of more substrates with several contaminated and non-contaminated soils. Once an appropriate set of substrates is determined, the modified MPN and MicroResp activity method could be tested as monitoring tool during a bioremediation project. Even if the MicroResp system works for a large number of soils it still remains to be conclusively proven that catabolic diversity is directly related to the functional diversity of the microbial community in a hydrocarbon-contaminated soil. Further open questions are

- whether VOC degraders decline as soon as volatile hydrocarbons disappear, and whether the modified MPN method is able to detect this decrease in numbers,
- whether appearance and disappearance of microbes detected with the MPN-method are also manifested in the CLPPs obtained with the MicroResp activity method,
- to which degree the CLPPs assessed with MicroResp activity are correlated with profiles of an RNA, DNA or PLFA based finger-printing methods such as DGGE, T-RFLP.

Further possible applications of the hybrid method or WST-method may be the screening of isolates for their capability to degrade certain volatile organic substances such as toluene, or to test (in soils or liquid cultures) how one substance influences the degradability of a second ^{14}C-labeled substance over a broad concentration range. This application may solve the debated question, whether ethanol as antiknock additive retards or hinders the degradation of the carcinogenic benzene in the vadose zone and groundwater after a fuel spill (41).

The subject concerning bioremediation and its pre-investigating and monitoring methods are by far not exhausted. Bioremediation techniques become more and more popular and are sometimes the only clean-up option particularly after large accidental spills, *e.g.* after an oil tanker has run aground, polluting hundreds of kilometers of shoreline (132). But also in midlands like Switzerland, remediation of new and old contaminated sites remains a challenge for engineers and scientists. It appears that we are presently unable to completely prevent new contaminations while older sites still wait to be remediated. The methods developed in the framework of this thesis will hopefully contribute to the understanding and optimization of bioremediation.

CHAPTER 7

References

1. **Abraham, M. H., J. Andonian-Haftvan, G. S. Whiting, A. Leo, and R. S. Taft.** 1994. Hydrogen bonding. Part 34. The factors that influence the solubility of gases and vapours in water at 298 K, and a new method for its determination. Journal of Chemical Society, Perkin Transactions 2**:**1777-1791.

2. **Admassu, W., and R. A. Korus.** 1998. Engineering of bioremediation processes: needs and limitations, p. 13-34. *In* R. L. Crawford and D. L. Crawford (ed.), Bioremediation: Principles and applications, 1st ed, vol. 6. Cambridge University Press, Cambridge, UK.

3. **Adriano, D. C., J.-M. Bollag, W. T. Frankenberger Jr., and R. C. Sims.** 1999. Preface. *In* D. C. Adriano, J.-M. Bollag, W. T. Frankenberger Jr., and R. C. Sims (ed.), Bioremediation of contaminated sites, vol. 37. American Society of Agronomy, Inc., Soil Science Society of America, Inc., Crop Science Society of America, Inc., Madison, Wisconsin.

4. **Adriano, D. C., A. Chlopecka, D. I. Kaplan, H. Clijesters, and J. Vangronsveld.** 1997. Soil contamination and remediation: Philosophy, science, and technology. 465-504. Presented at the 3rd International Conference on the Biogeochemistry of Trace Elements, Paris, R. Prost, INRA Press, Paris

5. **Ainsworth, C. C., F. J. Brockman, and P. M. Jardine.** 2000. Biogeochemical considerations and complexities, p. 829-923. *In* B. B. Looney and R. W. Falta (ed.), Vadose zone, science and technology solutions, vol. 2. Battelle Press, Columbus.

6. **Alexander, M.** 1999. Biodegradation and bioremediation, 2nd ed. Academic Press, San Diego.

7. **Alexander, M.** 1991. Introduction to soil microbiology. Wiley, New York and London.

8. **Allen-King, R. M., J. F. Barker, R. W. Gillham, and B. K. Jensen.** 1994. Substrate- and nutrient-limited toluene biotransformation in sandy soil. Environmental Toxicgoloy and Chemistry **13:**693-705.

9. **Allen-King, R. M., R. W. Gillham, J. F. Barker, and E. A. Sudicky.** 1996. Fate of dissolved toluene during steady infiltration through unsaturated soil: II. biotransformation under nutrient-limited conditions. Journal of Environmental Quality **25:**287-295.

10. **Ammann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews **59:**143-169.

11. **Anderson, J. P. E., and K. H. Domsch.** 1978. A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biology and Biochemistry **10:**215-221.

12. **Atlas, R. M.** 1979. Measurement of hydrocarbon biodegradation potentials and enumeration of hydrocarbon-utilizing microorganisms using carbon-14 hydrocarbon-spiked crude oil, p. 196-204. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology, vol. ASTM STP 695. American Society for Testing and Materials, West Conshohocken, PA, USA.

13. **Atlas, R. M.** 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiological Reviews **45:**180-209.

14. **Atlas, R. M., and R. Bartha.** 1992. Hydrocarbon biodegradation and oil spill bioremediation. Advances in Microbiological Ecology **12:**287-338.

15. **Bardgett, R. D., D. K. Leemans, R. Cook, and P. J. Hobbs.** 1997. Seasonality of the soil biota of grazed and ungrazed hill grasslands. Soil Biology and Biochemistry **29:**1285-1294.

16. **Becker, P. M., and W. Dott.** 1993. Miniaturisierte Screening-Tests zum Nachweis des mikrobiellen Abbaupotentials für verschiedene Aromaten und Aliphaten. BioEngineering **2/93:**20-26.

17. **Becker, P. M., and U. Stottmeister.** 1998. General (Biolog GN) versus site-relevant (pollutant-dependent) sole-carbon-source utilization patterns as a means to approaching community functioning. Canadian Journal of Microbiology **44:**913-919.

18. **Begon, M., J. L. Harper, and C. R. Townsend.** 1998. Oekologie. Spektrum Akademischer Verlag, Heidelberg and Berlin.

19. **Bekins, B., I. Cozzarelli, E. M. Godsy, E. Warren, H. I. Essaid, and M. E. Tuccillo.** 2001. Progression of natural attenuation processes at a crude-oil spill site: I I. Controls on spatial distribution of microbial populations. Journal of Contaminant Hydrology **53:**387-406.

20. **Beloin, R. M., J. L. Sinclair, and W. C. Ghiorse.** 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microbial Ecology **16:**85-97.

21. **Blanck, H.** 2002. A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. Human and Ecological Risk Assessment **8:**1003-1034.

22. **Bochner, B. R., and M. Savageau.** 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Applied and Environmental Microbiology **33:**434-444.

23. **Boivin, M. E. Y., A. M. Breure, L. Posthuma, and M. Rutgers.** 2002. Determination of field effects of contaminants - Significance of pollution-induced community tolerance. Human and Ecological Risk Assessment **8:**1035-1055.

24. **Bossio, D. A., and K. M. Scow.** 1995. Impact of carbon and flooding on the metabolic diversity of microbial communities in soils. Applied and Environmental Microbiology **61:**4043-4050.

25. **Bringmann, G., and R. Kühn.** 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Research **14:**231-241.

26. **Brockman, F. J., S. W. Li, J. K. Fredrickson, D. B. Ringelberg, T. L. Kieft, C. M. Spadoni, D. C. White, and J. P. McKinley.** 1998. Post-sampling changes in microbial community composition and activity in a subsurface paleosol. Microbial Ecology **36:**152-164.

27. **Brown, J. E., and J. F. Braddock.** 1990. Sheen Screen, a miniaturized most-probable-number method for enumeration of oil-degrading microorganisms. Applied and Environmental Microbiology **56:**3895-3896.

28. **Bundy, J. G., G. I. Paton, and C. D. Campbell.** 2002. Microbial communities in different soil types do not converge after diesel contamination. Journal of Applied Microbiology **92:**276-288.

29. **Buttler, A., P. Grosvernier, and Y. Matthey.** 1998. Development of *Sphagnum fallax* diaspores on bare peat with implications for restoration of cut-over bogs. Journal of Applied Ecology **35:**800-810.

30. **Campbell, C. D., S. J. Chapman, C. M. Cameron, M. S. Davidson, and J. M. Potts.** 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities using whole soil. Applied and Environmental Microbiology **69:**3593-3599.

31. **Campbell, C. D., S. J. Grayston, and D. J. Hirst.** 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. Journal of Microbiological Methods **30:**33-41.

32. **Caparello, D. M., and P. A. LaRock.** 1975. A radioisotope assay for the quantification of hydrocarbon biodegradation potential in environmental samples. Microbial Ecology **2:**28-42.

33. **Caplan, J. A.** 1993. The worldwide bioremediation industry: prospects for profit. Trends in Biotechnology **11:**320-323.

34. **Chapelle, F. H.** 1999. Bioremediation of petroleum hydrocarbon-contaminated ground water: The perspectives of history and hydrology. Ground Water **37:**122-132.

35. **Christophersen, M., and P. Kjeldsen.** 2001. Lateral gas transport in soil adjacent to an old landfill: Factors governing gas migration. Waste Management & Research **19:**579-594.

36. **Clement, B. G., L. E. Kehl, K. L. DeBord, and C. L. Kitts.** 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. Journal of Microbiological Methods **31:**135-142.

37. **Collins, A. M., J. M. Woodley, and J. M. Liddell.** 1995. Determination of reactor operation for microbial hydroxylation of toluene in a two liquid phase process. Journal of Industrial Microbiology **14:**382-388.

38. **Colwell, R. R.** 1979. Enumeration of specific populations by the most-probable-number (MPN) method, p. 56-61. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: Enumeration, activity, and ecology, vol. ASTM STP 695. American Society for Testing and Materials, West Conshohocken, PA, USA.

39. **Crawford, R. L.** 1998. Introduction, p. 1-12. *In* R. L. Crawford and D. L. Crawford (ed.), Bioremediation: Principles and Applications, 1st ed, vol. 6. Cambridge University Press, Cambridge, UK.

40. **CSO**. 1998. Contaminated sites ordinance; 26th August 1998. SR 814.680, Switzerland

41. **Dakhel, N., G. Pasteris, D. Werner, and P. Höhener.** 2003. Small-volume releases of gasoline in the vadose zone: Impact of the additives MTBE and ethanol on groundwater quality. Environmental Science & Technology **37:**2127-2133.

42. **Daugulis, A.** 1997. Partitioning bioreactors. Current Opinion in Biotechnology **8:**169-174.

43. **de Smet, M. J., J. Kingma, and B. Withold.** 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. Biochimica et Biophysica Acta **506:**64-80.

44. **Degens, B. P., and J. A. Harris.** 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biology and Biochemistry **29:**1309-1320.

45. **Derry, A. M., W. J. Staddon, and J. T. Trevors.** 1998. Functional diversity and community structure of microorganisms in uncontaminated and creosote-contaminated soils as determined by sole-carbon-source-utilization. World Journal of Microbiology and Biotechnology **14:**571-578.

46. **Deziel, E., Y. Comeau, and R. Villemur.** 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation **10:**219-233.

47. **Dibble, J. T., and R. Bartha.** 1979. Effect of environmental parameters on the biodegradation of oil sludge. Applied and Environmental Microbiology **37:**729-739.

48. **Dobbins, D. C., C. M. Aelion, and F. Pfaender.** 1992. Subsurface, terrestrial microbial ecology and biodegradation of organic chemicals: a review. Critical Reviews in Environmental Control **22:**67-136.

49. **Dobler, R., M. Saner, and R. Bachofen.** 2000. Population changes of soil microbial communities induced by hydrocarbon and heavy metal contamination. Bioremediation Journal **4:**41-56.

50. **Duarte, G. F., A. S. Rosado, L. Seldin, W. de Araujo, and J. D. van Elsas.** 2001. Analysis of bacterial community structure in sulfurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (dsz) genes. Applied and Environmental Microbiology **67:**1052-1062.

51. **Efroymson, R. A., and M. Alexander.** 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in non-aqueous phase liquids. Environmental Science & Technology **29:**515-521.

52. **Efroymson, R. A., and M. Alexander.** 1994. Role of partitioning in biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. Environmental Science & Technology **28:**1172-1179.

53. **Ekelund, F., R. Rønn, and S. Christensen.** 2001. Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites. Soil Biology & Biochemistry **33:**475-481.

54. **Ellingsøe, P., and K. Johnsen.** 2002. Influence of soil sample size on the assessment of bacterial community structure. Soil Biology & Biochemistry **34:**1701-1707.

55. **Ellis, R. J., B. Neish, M. W. Trett, J. G. Best, A. J. Weightman, P. Morgan, and J. C. Fry.** 2001. Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. Journal of Microbiological Methods **45:**171-185.

56. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Applied and Environmental Microbiology **57:**1139-1145.

57. **Eweis, J. B., S. J. Ergas, D. P. Y. Chang, and E. D. Schroeder.** 1998. Bioremediation principles. WCB McGraw Hill, Davis, USA.

58. **Fent, K.** 2003. Oekotoxikologie, 2nd ed. Thieme, Stuttgart.

59. **Fliermans, C. B., M. M. Franck, T. C. Hazen, and R. W. Gorden.** 1997. Ecofunctional enzymes of microbial communities in ground water. FEMS Microbiology Reviews **20:**379-389.

60. **Freijer, J. I., H. de Jonge, W. Bouten, and J. M. Verstraten.** 1996. Assessing mineralization rates of petroleum hydrocarbons in soils in relation to environmental factors and experimental scale. Biodegradation **7:**487-500.

61. **Garland, J. L.** 1996. Patterns of potential C source utilization by rhizosphere communities. Soil Biology & Biochemistry **28:**223-230.

62. **Garland, J. L., and A. L. Mills.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology **57:**2351-2359.

63. **Garnier, P. M., R. Auria, C. Augur, and S. Revah.** 1999. Cometabolic biodegradation of methyl t-butyl ether by *Pseudomonas aeruginosa* grown on pentane. Applied Microbiology and Biotechnology **51:**498-503.

64. **Ghoshal, S., A. Ramaswami, and R. Luthy.** 1996. Biodegradation of naphtalene from coal tar and heptamethylnonane in mixed batch systems. Environmental Science & Technology **30:**1282-1291.

65. **Gibson, D. T.** 1984. Microbial degradation of organic compounds. Marcel Dekker, New York.

66. **Gorden, R. W., T. C. Hazen, and C. B. Fliermans.** 1993. Rapid screening method for bacteria capable of degrading toxic organic compounds. Journal of Microbiological Methods **18:**339-347.

67. **Grathwohl, P., D. Halm, A. Bonilla, M. Broholm, V. Burganos, M. Christophersen, R. Comans, P. Gaganis, I. Gorostiza, P. Höhener, P. Kjeldsen, and H. van der Sloot.** 2003. Guideline for groundwater risk assessement at contaminated sites (GRACOS). Energy, Environment and Sustainable Development Programme of the European Commission implemented under the Fifth Framework Programme. http://www.uni-tuebingen.de/gracos/.

68. **Greene, E. A., J. G. Kay, K. Jaber, L. G. Stehmeier, and G. Voordouw.** 2000. Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. Applied and Environmental Microbiology **66:**5282-5289.

69. **Gremion, F., A. Chatzinotas, K. Kaufmann, W. von Sigler, and H. Harms.** in press. Impacts of heavy metal contamination and phytoremediation on the microbial community during a twelve months microcosm experiment. FEMS Microbiology Ecology.

70. **Griffiths, R. I., A. S. Whiteley, A. G. O'Donnell, and M. J. Bailey.** 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Applied and Environmental Microbiology **66:**5488-91.

71. **Haack, S. K., H. Garchow, M. J. Klug, and L. J. Forney.** 1995. Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization pattern. Applied and Environmental Microbiology **61:**1458-1468.

72. **Haider, K.** 1999. Microbe-soil-organic contaminant interaction. *In* D. C. Adriano, J.-M. Bollag, W. T. Frankenberger Jr., and R. C. Sims (ed.), Bioremediation of contaminated sites, vol. 37. American Society of Agronomy, Inc., Soil Science Society of America, Inc., Crop Science Society of America, Inc., Madison, Wisconsin, USA.

73. **Häner, A., P. Höhener, and J. Zeyer.** 1997. Degradation of trimethylbenzene isomers by an enrichment culture under N_2O-reducing conditions. Applied and Environmental Microbiology **63:**1171-1174.

74. **Hansch, C., A. Leo, and D. Hoekman.** 1995. Exploring QSAR - Hydrophobic, electronic, and steric constants. American Chemical Society, Washington DC.

75. **Harris, D.** 1994. Analysis of DNA extracted from microbial communities, p. 111-118. *In* K. Ritz, J. Dighton, and K. E. Giller (ed.), Beyond the biomass, compositional and functional analysis of soil microbial communities. Wiley, Chichester.

76. **Heinonsalo, J., K. S. Jørgensen, K. Haahtela, and R. Sen.** 2000. Effects of *Pinus sylvestris* root growth and mycorrhizosphere development on bacterial carbon source utilization and hydrocarbon oxidation in forest and petroleum-contaminated soils. Canadian Journal of Microbiology **46:**451-464.

77. **Heitzer, A., and G. S. Sayler.** 1993. Monitoring the efficacy of bioremediation. Trends in Biotechnology **11:**334-343.

78. **Heuer, H., and K. Smalla.** 1997. Evaluation of community-level catabolic profiling using Biolog GN microplates to study microbial community changes in potato phyllosphere. Journal of Microbiological Methods **30:**49-61.

79. **Höhener, P., C. Duwig, G. Pasteris, K. Kaufmann, N. Dakhel, and H. Harms.** 2003. Biodegradation of petroleum hydrocarbon vapors: laboratory studies on rates and kinetics in unsaturated alluvial sand. Journal of Contaminant Hydrology **66:**93-115.

80. **Höhener, P., D. Hunkeler, A. Hess, T. Bregnard, and J. Zeyer.** 1998. Methodology for the evaluation of engineered *in situ* bioremediation: lessons from a case study. Journal of Microbiological Methods **32:**179-192.

81. **Hopkins, D. W., S. J. MacNaughton, and A. G. O'Donnell.** 1991. A dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. Soil Biology & Biochemistry **23:**217-225.

82. **Hornick, S. B., R. H. Fisher, and P. A. Paolini.** 1983. Petroleum wastes, p. 321-337. *In* J. F. Parr, P. B. Marsh, and J. M. Kla (ed.), Land treatment of hazardous wastes. Noyes Data Corp., Park Ridge, NJ, USA.

83. **Howard, P. J. A.** 1997. Correspondence. Soil Biology and Biochemistry **29:**1755-1757.

84. **Hubert, C., Y. Shen, and G. Voordouw.** 1999. Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. Applied and Environmental Microbiology **65:**3064-3070.

85. **Hurley, M. A., and M. E. Roscoe.** 1983. Automated statistical analysis of microbial enumeration by dilution series. Journal of Applied Microbiology **55:**159-164.

86. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentrations of toluene. Nature **338:**264-266.

87. **Insam, H., K. Amor, M. Renner, and C. Crepaz.** 1996. Changes in functional abilities of the microbial community during composting of manure. Microbial Ecology **31:**77-87.

88. **Insam, H., and A. Rangger.** 1997. Microbial communities - functional versus structural approaches. Springer Verlag, Berlin Heidelberg.

89. **Ishiyama, M., M. Shiga, K. Sasamoto, M. Mizoguchi, and P. He.** 1993. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chemical and Pharmaceutical Bulletin **41:**1118-1122.

90. **Johnsen, A. R., K. Bendixen, and U. Karlson.** 2002. Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates using the respiration indicator WST-1. Applied and Environmental Microbiology **68:**2683-2689.

91. **Jordi, B.** 2003. Sauberes Grundwasser. Swiss Agency for the Landscape, Forests and Environment (SAFEL).

92. **Jordi, B.** 2003. Teure Aufräumaktion zum Schutz der Gesundheit. Swiss Agency for the Landscape, Forests and Environment (SAFEL).

93. **Katsivela, E., E. R. B. Moore, and N. Kalogerakis.** 2003. Biodegradation of aliphatic and aromatic hydrocarbons: specificity among bacteria isolated from refinery waste sludge. Water, Air and Soil Pollution: Focus **3:**103-115.

94. **Kaufmann, K., S. J. Chapman, C. D. Campbell, H. Harms, and P. Höhener.** submitted. Rapid testing of volatile petroleum hydrocarbon degradation in nutrient amended soils using a micro-respiration system. Applied and Environmental Microbiology.

95. **Kellner, R. A., J.-M. Mermet, and M. Otto.** 1998. Analytical Chemistry. Wiley-VHC, Weinheim, Germany.

96. **Kersters, I., L. Van Vooren, L. Verschuere, L. Vauterin, A. Wouters, J. Mergaert, J. Swings, and W. Verstraete.** 1997. Utility of the Biolog system for the characterization of heterotrophic microbial communities. Systematic and Applied Microbiology **20:**439-447.

97. **Kjeldsen, P., M. Christophersen, M. Broholm, P. Höhener, R. Aravena, and D. Hunkeler.** 2003. Biodegradation of fuel vapours in the vadose zone at Airbase Værløse, Denmark. 31-39. Presented at the 2nd International Workshop on Groundwater Risk Assessment at Contaminated Sites and Integrated Soil and Water Protection, Tuebingen, P. Grathwohl, Tübinger Geowissenschaftliche Arbeiten Vol. 69

98. **Konopka, A., L. Oliver, and R. F. Turco Jr.** 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microbial Ecology **35:**103-115.

99. **Konopka, A., and R. Turco.** 1991. Biodegradation of organic compounds in the vadose zone and aquifer sediments. Applied and Environmental Microbiology **57:**2260-2268.

100. **Korda, A., P. Santas, A. Tenente, and R. Santas.** 1997. Petroleum hydrocarbon bioremediation: sampling and analytical techniques, *in situ* treatments and commercial microorganisms currently used. Applied Microbiology and Technology **48:**677-686.

101. **Kozdroj, J., and J. D. van Elsas.** 2001. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. Journal of Microbiological Methods **43:**197-212.

102. **Kunze, K. D.** 1979. Bedeutung der Redoxmediatoren für den histochemischen Nachweis von Dehydrogenasen, p. 166-183. *In* F. E. Wohlrab, E. Seidler, and D. J. A. Kunze (ed.), Histo- und Zytochemie dehydrierender Enzyme. Grundlagen und Problematik. Barth, Leipzig, Germany.

103. **Legendre, P., and L. Legendre.** 1998. Numerical ecology, Second english edition ed, vol. 20. Elsevier Science B.V., Amsterdam.

104. **Looney, B. B., and R. W. Falta.** 2000. The vadose zone, what is it, why it matters, and how it works, p. 3-59. *In* B. B. Looney and R. W. Falta (ed.), Vadose zone, science and technology, vol. 1. Battelle Press, Columbus.

105. **MacNaughton, S. J., J. R. Stephen, A. D. Venosa, G. A. Davis, Y.-J. Chang, and D. C. White.** 1999. Microbial population changes during bioremediation of an experimental oil spill. Applied and Environmental Microbiology **65:**3566-3574.

106. **Maier, U., U. Mayer, and P. Grathwohl.** 2003. Modelling transport and natural attenuation of fuel compounds in the vadose zone at the emplaced fuel source experiment, Airbase Værløse, Denmark. 47-52. Presented at the 2nd International Workshop on Grounwater Risk Assessment at Contaminated Sites and Integrated Soil and Water Protection, Tübingen, Germany, D. Halm and P. Grathwohl,

107. **Marshall, K. C.** 1976. Interfaces in microbial ecology. Harvard University Press, London.

108. **Marstorp, H., and E. Witter.** 1999. Extractable dsDNA and product formation as measures of microbial growth in soil upon substrate addition. Soil Biology and Biochemistry **31:**1443-1453.

109. **Mayer, P., J. Wernsing, J. Tolls, P. G.-J. de Maagd, and D. T. H. M. Sijm.** 1999. Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. Environmental Science & Technology **33:**2284-2290.

110. **Mayr, C., M. Miller, and H. Insam.** 1999. Elevated CO_2 alters community-level physiological profiles and enzyme activities in alpine grassland. Journal of Microbiological Methods **36:**35-43.

111. **McCarty, P. L.** 1991. Engineering concepts for *in situ* bioremediation. Journal of Hazardous Materials **28:**1-11.

112. **McLean, V. A.** 1985. Handbook of remedial action at waste disposal sites EPA/ 625/6-85/006. U.S. EPA.

113. **Mills, A. L., C. Breuil, and R. R. Colwell.** 1978. Enumeration of petroleum-degrading marine and estuarine microorganisms by the most probable number method. Canadian Journal of Microbiology **24:**552-557.

114. **Mills, S. A., and W. T. Frankenberger Jr.** 1994. Evaluation of phosphorus sources promoting bioremediation of diesel fuel in soil. Bulletin of Environmental Contaminant Toxicology **53:**280-284.

115. **Moran, M. D.** 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos **100:**403-405.

116. **Morrison, D. E., and M. Alexander.** 1997. Biodegradability of nonaqueous phase liquids affects the mineralisation of phenanthrene in soil because of microbial competition. Environmental Toxicoloy and Chemistry **16:**1561-1567.

117. **Naeem, S., and S. B. Li.** 1997. Biodiversity enhances ecosystem reliability. Nature **390:**507-509.

118. **Naeem, S., L. J. Thompson, S. P. Lawler, J. H. Lawton, and R. M. Woodfin.** 1994. Declining biodiversity can alter the performance of ecosystems. Nature **368:**734-737.

119. **Odu, C. T. I.** 1972. Microbiology of soils contaminated with petroleum hydrocarbons. I. Extent of contamination and some soil and microbial properties after contamination. Journal of the Institute of Petroleum **58:**200-208.

120. **OIS**. 1998. Swiss ordinance relating to impacts on the soil; 1st July 1998. SR 814.12, Switzerland

121. **Orzech, M., J. Solyst, and P. Thompson.** 1991. State's use of bioremediation: advantages, constraints, and strategies. Centre of Policy Research, National Governor's Association.

122. **Park, H. P., A. Hussam, P. Couasnon, D. Fritz, and P. W. Carr.** 1987. Experimental reexamination of selected partition coefficients from Rohrschneider's data set. Analytical Chemistry **59:**1970-1976.

123. **Pasteris, G., D. Werner, K. Kaufmann, and P. Höhener.** 2002. Vapor phase transport and biodegradation of volatile fuel compounds in the unsaturated zone: a large scale lysimeter experiment. Environmental Science & Technology **36:**30-39.

124. **Poe, S. H., K. T. Valsaraj, L. J. Thibodeaux, and C. Springer.** 1988. Equilibrium vapour phase adsorption of volatile organic chemicals on dry soils. Journal of Hazardous Materials **19:**17-32.

125. **Preston-Mafham, J., L. Boddy, and P. F. Randerson.** 2002. Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. FEMS Microbiology Ecology **42:**1-14.

126. **Pucci, O. H., M. A. Bak, S. R. Peressutti, I. Klein, C. Härtig, H. M. Alvarez, and L. Wünsche.** 2000. Influence of crude oil contamination on the bacterial community of semarid soils of Patagonia (Argentina). Acta Biotechnologica **20:**129-146.

127. **Raap, A. K., and P. Van Dujin.** 1983. Studies on the metosulphate-tetrazolium capture reaction $NAD(P)^+$-dependent dehydrogenase cytochemistry. II. A novel hypothesis for the mode of action of PMS and a study of the properties of reduced PMS. Histochemical Journal **15:**881-893.

128. **Riser-Roberts, E.** 1992. Bioremediation of petroleum contaminated sites. CRC Press, Boca Raton, Florida.

129. **Riser-Roberts, E.** 1998. Remediation of petroleum contaminated soils - biological, physical, and chemical processes. Lewis Publishers, Boca Raton, Florida.

130. **Röling, W. F. M., M. G. Milner, D. M. Jones, K. Lee, F. Daniel, R. J. P. Swannell, and I. M. Head.** 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Applied and Environmental Microbiology **68:**5537-5548.

131. **Rontani, J. F., and G. Giusti.** 1986. Short Note - Study of the biodegradation of poly-branched alkanes by a marine bacterial community. Marine Chemistry **20:**197-205.

132. **Rosenberg, E., and E. Z. Ron.** 1998. Bioremediation of petroleum contamination, p. 100-124. *In* R. L. Crawford and D. L. Crawford (ed.), Bioremediation: Principles and Applications, vol. 6. Cambridge University Press, Cambridge.

133. **Roubal, G., and R. M. Atlas.** 1978. Distribution of hydrocarbon-utilizing microorganisms and hydrocarbon biodegradation potentials in Alaskan continental shelf areas. Applied and Environmental Microbiology **35:**897-905.

134. **Rutter, P. R., and B. Vincent.** 1980. The adhesion of micro-organisms to surfaces: physio-chemical aspects., p. 79-92. *In* J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Society of Chemical Industry, London.

135. **Schachtschabel, P., H.-P. Blume, G. W. Brümmer, K.-H. Hartge, and U. Schwertmann.** 1998. Lehrbuch der Bodenkunde, 14th ed. Ferdinand Enke Verlag, Stuttgart, Germany.

136. **Schaeffer, T. L., S. G. Cantwell, J. L. Brown, D. S. Watt, and R. R. Fall.** 1979. Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. Applied and Environmental Microbiology **38:**742-746.

137. **Schraa, G., B. M. Bethe, A. R. W. van Neerven, W. J. van den Tweel, E. van der Wende, and A. J. B. Zehnder.** 1987. Degradation of 1,2-dimethylbenzene by *Corynebacterium* strain C125. Antonie van Leeuwenhoek **53:**159-170.

138. **Schwarzenbach, R. P., P. M. Gschwend, and D. M. Imboden.** 2003. Environmental organic chemistry, 2nd ed. Wiley, Hoboken, New Jersey.

139. **Schwarzenbach, R. P., and J. Westall.** 1981. Transport of nonpolar organic compounds from surface water to groundwater. Laboratory sorption studies. Environmental Science & Technology **15:**1360-1367.

140. **Shi, W., J. Becker, M. Bischoff, R. F. Turco, and A. E. Konopka.** 2002. Association of microbial community composition and activity with lead, chromium, and hydrocarbon contamination. Applied and Environmental Microbiology **68:**3859-3866.

141. **Siciliano, S. D., and J. J. Germida.** 1998. Biolog analysis and fatty acid methyl ester profiles indicate that pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*. Soil Biology and Biochemistry **30:**1717-1723.

142. **Song, H.-G., and R. Bartha.** 1990. Effects of jet fuel spills on the microbial community of soil. Applied and Environmental Microbiology **56:**646-651.

143. **Song, H.-G., T. A. Pedersen, and R. Bartha.** 1985. Hydrocarbon mineralization in soil: relative bacterial and fungal contribution. Soil Biology & Biochemistry **18:**109-111.

144. **Spain, J. C., P. H. Pritchard, and A. W. Bourquin.** 1980. Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. Applied and Environmental Microbiology **40:**726-734.

145. **Stephens, D. B.** 1996. Vadose zone hydrology. Lewis Publishers Inc., Boca Raton, USA.

146. **Strong-Gunderson, J. M., and A. V. Palumbo.** 1994. Alternative method for rapidly screening microbial isolates for their potential to degrade volatile contaminants. Journal of Industrial Microbiology **13:**361-366.

147. **Taylor, J. P., B. Wilson, M. S. Mills, and R. G. Burns.** 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biology & Biochemistry **34:**387-401.

148. **ter Braak, C. J. F.** 1988. CANOCO-a FORTRAN program for Canonical Community Ordination by Partial, Detrended or Canonical Correspondence Analysis, Principal Components Analysis and Redundancy Analysis, 2.1 ed. Agricultural Mathematics Group, Wageningen, the Netherlands.

149. **ter Braak, C. J. F.** 1990. Update Notes: CANOCO Version 3.10. Agricultural Mathematics Group, Wageningen, the Netherlands.

150. **Thom, S. M., R. W. Horobin, E. Seidler, and M. R. Barer.** 1993. Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. Journal of Applied Bacteriology **74:**433-443.

151. **Thomas, J. M., and C. H. Ward.** 1989. *In situ* biorestoration of organic contaminants in the subsurface. Environmental Science & Technology **23:**760-766.

152. **Thompson, L. M., C. A. Black, and J. A. Zoellner.** 1954. Occurence and mineralization of organic phosphorous in soils, with particular reference to associations with nitrogen, carbon, and pH. Soil Science **77:**185-196.

153. **Thornton-Manning, J. R., D. D. Jones, and T. W. Federle.** 1987. Effects of experimental manipulation of environmental factors on phenol mineralization in soil. Environmental Toxicoloy and Chemistry **6:**615-621.

154. **Torsvik, V., L. Øvreås, and T. F. Thingstad.** 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science **296:**1064-1066.

155. **Torsvik, V., K. Salte, R. Sørheim, and J. Goksøyr.** 1990. Comparison of phenotypic diversity and DNA heterogeneity population of soil bacteria. Applied and Environmental Microbiology **56:**776-781.

156. **Torsvik, V., R. Sørheim, and J. Goksøyr.** 1996. Total bacterial diversity in soil and sediment communities - A review. Journal of Industrial Microbiology **17:**170-178.

157. **Turco, R. F.** 1999. Soil microorganisms and their requirements, p. 15-32. *In* D. C. Adriano, J.-M. Bollag, W. T. Frankenberger Jr., and R. C. Sims (ed.), Bioremediation of contaminated soils, vol. 37. American Society of Agronomy, Inc., Soil Science Society of America, Inc., Crop Science Society of America, Inc., Madison, Wisconsin, USA.

158. **U.S. National Library of Medicine** January 2004. TOXNET. U. S. National Library of Medecine, Bethesda, MD. http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~3ITWeR:1.

159. **van Beelen, P.** 2003. A review on the application of microbial toxicity tests for deriving sediment quality guidelines. Chemosphere **53:**795-808.

160. **Verschuere, L., V. Fievez, L. Van Vooren, and W. Verstraete.** 1997. The contribution of individual populations to the Biolog pattern of model microbial communitites. FEMS Microbiology Ecology **24:**353-362.

161. **Waksman, S. A.** 1924. Influence of microorganisms upon the carbon:nitrogen ratio in the soil. The Journal of Agricultural Science **14:**555-562.

162. **Walker, J. D., and R. R. Colwell.** 1976. Enumeration of petroleum-degrading microorganisms. Applied and Environmental Microbiology **31:**198-207.

163. **Wardle, D. A., and K. E. Giller.** 1996. The quest for a contemporary ecological dimension to soil biology. Soil Biology & Biochemistry **28:**1549-1554.

164. **Werner, D.** 2002. Gaseous tracer diffusion from a point source as a site investigation method. PhD Thesis No. 2625. École polytechnique fédéral de Lausanne, Lausanne, Switzerland.

165. **White, D. C., C. A. Flemming, K. T. Leung, and S. J. MacNaughton.** 1998. *In situ* microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere and in biofilms. Journal of Microbiological Methods **32:**93-105.

166. **Widmer, F., A. Fliessbach, E. Laczko, J. Schulze-Aurich, and J. Zeyer.** 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PFLA-, and Biolog-analyses. Soil Biology & Biochemistry **33:**1029-1036.

167. **Wiedemeier, T. H., H. S. Rifai, C. J. Newell, and J. T. Wilson.** 1999. Natural attenuation of fuels and chlorinated solvents in the subsurface. Wiley, New York.

168. **Winding, A.** 1994. Fingerprinting bacterial soil communities using Biolog microtitre plates, p. 85-94. *In* K. Ritz, J. Dighton, and K. E. Giller (ed.), Beyond the biomass. Wiley, New York.

169. **Winding, A., and N. B. Hendriksen.** 1997. Biolog substrate utilisation assay for metabolic fingerprints of soil bacteria: incubation effects, p. 195-205. *In* H. Insam and A. Rangger (ed.), Microbial communities: functional versus structural approaches, 1 ed. Springer, Heidelberg.

170. **Wodzinski, R. S., and D. Larocca.** 1977. Bacterial growth kinetics on diphenylmethane and naphtalene-heptamethylnonane mixtures. Applied and Environmental Microbiology **33:**660-665.

171. **Wrenn, B. A., and A. D. Venosa.** 1996. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable number procedure. Canadian Journal of Microbiology **42:**252-258.

172. **Wünsche, L., L. Brüggemann, and W. Babel.** 1995. Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution. FEMS Microbiology Ecology **17:**295-306.

173. **Zarda, B., G. Mattison, A. Hess, D. Hahn, P. Höhener, and J. Zeyer.** 1998. Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. FEMS Microbiology Ecology **27:**141-152.

174. **Zeng, Y.** Toluene pathway map. University of Minnesota. http:// umbbd.ahc.umn.edu/tol/tol_map.html.

175. **Zhou, E., and R. L. Crawford.** 1995. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. Biodegradation **6:**127-140.

176. **Zhou, J., B. Xia, D. S. Trevers, L. Y. Wu, T. L. Marsh, R. V. O'Neill, A. V. Palumbo, and J. M. Tiedje.** 2002. Spatial and resource factors influencing high microbial diversity in soil. Applied and Environmental Microbiology **68:**326-334.

177. **ZoBell, C. E.** 1946. Action of microorganisms on hydrocarbons. Bacteriological Reviews **10:**1-49.

APPENDICES

Appendix I: Calculation of hydrocarbon concentrations in the aqueous phase

The Raoult's law describes the partitioning of organic compounds from organic liquid mixtures into air under consideration of vapor pressures over the pure compounds. Briefly, the molar concentration of a compound *i* of a hydrocarbon mixture in the air at equilibrium ($C_{i\,air}$) can be calculated as follows (138):

$$C_{i\,air} = \frac{x_{i\,mix} \cdot \gamma_{i\,mix}}{\gamma_{i\,air} \cdot V_{air}} \qquad \text{(eq. I.1)}$$

One needs to know the molefraction $x_{i\,mix}$ of the compound in a carrier phase-substrate mixture as well as its activity coefficients in the organic ($\gamma_{i\,mix}$) and in the gaseous ($\gamma_{i\,air}$) phases. If the mixture contains no important amount of polar compounds and if big salt concentrations in the system can be excluded (for details see (138)) and hence neither co-solvent nor salt effects occur, $(\gamma_{i\,\mathrm{air}}\, V_{\mathrm{air}})^{-1}$ can be substituted by the *liquid* compounds saturation concentration in the gaseous phase $C^{sat}_{i\cdot air} \cdot \gamma_{i\,mix}$-values are expected to be around 1 for a) apolar compounds in mixtures in which the major components primarily undergo vdW interactions, and b) monopolar compounds in the same situation, if there is no major constituent in the mixture exhibiting a significant complementary polarity. The above equation can then be simplified to:

$$C_{i\,air} = x_{i\,mix} \cdot C^{sat}_{i\,air} \qquad \text{(eq. I.2)}$$

The resulting theoretical aqueous concentrations of hydrocarbons in water are calculated with Henry's law. Examples meeting criteria for $\gamma_{i\,mix} = 1$ are BTEX compounds, aliphatic hydrocarbons and MTBE, but excluded are compounds such as certain antiknock additives to gasoline, *e.g.* ethanol. For ethanol $\gamma_{i\,mix}$=35 (122) was used. Final water concentrations are calculated with equation 3:

$$C_{i\,water} = \frac{C_{i\,air}}{H_i} \qquad \text{(eq. I.3)}$$

Appendix II: List of abbreviations and notations

μ	Growth rate
$\gamma_{i\ air}$	Activity coefficient of compound *i* in the air phase
$\gamma_{i\ mix}$	Activity coefficient of compound *i* in the organic phase
μ_{max}	Maximal growth rate
1,2,4-TMB	1,2,4-Trimethylbenzene
ANOVA	Analysis of variance
AO	Acridine orange
AS	Assebroek, Belgium
ATD	Automated thermal desorption
AWCD	Average well colour development
Bq	Becquerel
BSA	Bovine serum albumin
BTEX	Benzene, toluene, ethylbenzene, xylenes
$C^{sat}_{i\ air}$	Saturation concentration of compound *i* in the air
$C_{\ i\ (air)}$	Concentration of compound *i* in the air
CFC	Chlorofluorocarbon
C_i^{sat}	Maximum water solubility of compound *i*
CLPP	Community-level physiological profile
CSO	Contaminated Sites Ordinance
CV	Coefficient of variation
CWR	Countesswells Road, Aberdeen, Scotland
DA	Discriminant analysis
DAPI	4',6'-diamino-2-phenylindole hydrochloride
DDT	1,1,1-Trichlor-2,2-bis(p-chlorphenyl)ethane
DGGE	Denaturing gradient gel electrophoresis
EC_{50}	Effect concentration, concentration with defined effect on 50 % of test organisms
ECD	Electron capture detection
EtOH	Ethanol
F	Result of F-test

FID	Flame ionization detector
GC	Gas chromatograph
GRACOS	Groundwater risk assessment at contaminated sites (European project)
HCarb	Hydrocarbon
HDPP	High density polypropylene
H_i	Air water partitioning coefficient (Henry constant)
HMN	2,2,4,4,6,8,8-heptamethylnonane
ICP-AES	Inductively coupled plasma – atomic emission spectroscopy
INT	Iodonitrotetrazolium violet
Isooct	Isooctane
$K_{air\text{-}hex}$	Partitioning coefficient between air and hexadecane
$K_{hex\text{-}water}$	Hexadecane-water partitioning coefficient
K_I	Inhibition constant
K_{OC}	Organic carbon-water partitioning coefficient
K_{OW}	Octanol-water partitioning coefficient
K_S	Saturation constant
LC_{50}	Lethal concentration, concentration with lethal effect on 50 % of test organisms
LL	Lower limit
MCH	Methyl-cyclohexane
Mix	Mixture
MPN	Most probable number
MTBE	Methyl *tert*-butyl ether
MW_i	Molecular weight of compound *i*
$NADH_2$	Nicotinamide adenine dinucleotide, reduced form
NAPL	Non-aqueous phase liquid
No	Number
N_{tot}	Total nitrogen
Oct	*n*-Octane
OD	Optical density
OIS	Ordinance Relating to Impacts on the Soil
p	Probability

PAH	Polycyclic aromatic hydrocarbon
PCA	Principal component analysis
PCB	Polychlorinated biphenyls
PCE	Perchloroethene, Tetrachloroethene
PE	Polyethylene
Permut	Permutation test
PICT	Pollution induced community tolerance
PLFA	Phospholipid fatty acid
PP	Polypropylene
Prot	Protein
PTFE	Polytetrafluoroethylene
P_{tot}	Total phosphorous
PVC	Polyvinyl chloride
RDA	Redundancy analysis
RFLP	Restriction fragment length polymorphism
S	Substrate richness
S	Nutrient concentration
T	Temperature
TC	Total carbon
TCD	Thermal conductivity detector
TCE	Trichloroethene
TGGE	Temperature gradient gel electrophoresis
TOC	Total organic carbon
Tol	Toluene
T-RFLP	Terminal restriction fragment length polymorphisms
UL	Upper limit
V_{air}	Air volume
VC	Vinylchloride
vdW	Van der Waal
VOC	Volatile organic carbon
Wat Cont	Water Content
WHC	Water holding capacity
wt	Weight
$x_{i\,mix}$	Molefraction of compound i in hydrocarbon mixture

λ	Eigenvalue
θ_w	Water-filled porosity
WST-1	Water soluble tetrazolium 1

Appendix III: List of figures

Appendix IV: List of tables

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Publications

Kaufmann, K., Chapman, S.J., Campbell, C.D., Harms, H., and Höhener, P. (in prep) Soil microbial community response to hydrocarbon pollution assessed using a micro-res-

piration method incorporating a two-liquid phase system to control volatile substrate concentration. *Environmental Microbiology*.

Kaufmann, K., Chapman, S.J., Campbell, C.D., Harms, H., and Höhener, P. (submitted) Rapid testing of volatile petroleum hydrocarbon degradation in nutrient amended soils using a micro-respiration system. *Environmental Microbiology*.

Kaufmann, K., Christophersen, M., Buttler, A., Harms, H., and Höhener, P. (in press) Microbial community response to petroleum hydrocarbon contamination in the unsaturated zone at the experimental field site Værløse, Denmark. *FEMS Microbiology Ecology*.

Gremion, F., Chatzinotas, A., Kaufmann, K., von Sigler, W., and Harms, H. (in press) Impacts of heavy metal contamination and phytoremediation on the microbial community during a twelve months microcosm experiment. *FEMS Microbiology Ecology*.

Hoehener, P., Duwig, C., Pasteris, G., Kaufmann, K., Dakhel, N., and Harms, H. (2003) Biodegradation of petroleum hydrocarbon vapors: laboratory studies on rates and kinetics in unsaturated alluvial sand. *Journal of Contaminant Hydrology* **66**: 93-115.

Pasteris, G., Werner, D., Kaufmann, K., and Höhener, P. (2002) Vapor phase transport and biodegradation of volatile fuel compounds in the unsaturated zone: a large scale lysimeter experiment. *Environmental Science & Technology* **36**: 30-39.

Medilanski, E., Kaufmann, K., Wick, L.Y., Wanner, O., and Harms, H. (2002) Influence of the surface topography of stainless steel on bacterial adhesion. *Biofouling* **18**: 193-203.

Volkland, H.-P., Harms, H., Kaufmann, K., Wanner, O., and Zehnder, A.J.B. (2001) Repair of damaged vivianite coatings on mild steel using bacteria. *Corrosion Science* **43**: 2135-2146