

# Effect of N and P fertilisation and aeration on biodegradation of crude oil in aged hydrocarbon contaminated soils

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## ABSTRACT

We conducted two laboratory experiments to examine the effects of fertilisation and agitation (aeration) on crude oil degradation in two soils with differential nutrient (nitrogen, phosphorus) availability. Two soils that had been spiked with crude oil two years before were mixed with nitrogen and/or phosphorus at three different levels and subsequently incubated 28 days (Exp. 1). In experiment 2 we investigated the effect of repeated agitation (manual mixing) on hydrocarbon degradation with and without fertilisation. One of the soils was also freshly spiked to assess the impact of ageing. Heptane-extractable hydrocarbon concentrations were determined in both experiments and substrate-induced respiration in Exp. 2. The generally small changes of hydrocarbon concentrations during 28 days of incubation in Exp. 1 are likely attributed to low bio-accessibility of hydrocarbons as a consequence of long-term ageing. Fertilization of nitrogen, phosphorus or combinations thereof was ineffective in most treatments of Exp. 1, which may be explained by limited oxygen supply due to the high proportions of clay and silt. However, agitation enhanced HC biodegradation in the sandy-loamy soil by about 15% (Exp. 2) compared to non-agitated treatments. In contrast, we observed no effect of agitation in the sandy soil.

**Keywords:** biodegradation; crude oil; hydrocarbon; fertiliser; contaminated soils; incubation

Contamination of soils with crude oil deriving from e.g. exploration and processing of petroleum is of major environmental concern. The various compounds of crude oil are used to produce products such as gasoline, kerosene propane, fuel oil, lubricating oil, wax, and asphalt (Atlas 1995). Based on the nature of the hydrocarbons (HC), crude oil can be classified into three groups: (i) paraffin-based crude oils; (ii) asphaltic-base crude oils and (iii) mixed-base crude oils (Cleveland 2007). Additionally, petroleum contains a complex mixture of compounds which can be categorized into four fractions: saturates, aromatics, resins and asphaltenes (Killops and Al-Jaboori 1990, Outdot et al. 1998).

In several oil-producing countries, crude oil is one of the most important contaminants of soils

and sediments. Large areas have been contaminated with petroleum oil near the oil refineries and oil storage tanks. Crude oil may cause land degradation by affecting plants and soil micro-organism. These harmful effects depend on the concentration and type of contaminant (Boethling and Alexander 1979).

Bioremediation techniques have become a major tool to enhance natural biodegradation of crude oil residuals (based on indigenous microorganisms). Among the different techniques to enhance natural biodegradation by indigenous microorganisms, inoculation with hydrocarbon-degrading bacteria and fertilizing the indigenous population have attracted the most interest. The application of nutrients such as nitrogen and phosphorous was shown to be most effective in accelerating the

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biodegradation process and at the same time to be environmentally safe. Nitrogen and phosphorus are usually reported to be limiting factors of hydrocarbon degradation in oxic soil environments (Pritchard and Costa 1991, Bragg et al. 1994, Wright et al. 1997, Margesin et al. 2000, Van Hamme et al. 2003).

Some reports are available on the influence of fertiliser application on degradation of hydrocarbons. Lin et al. (1999) reported that fertiliser application in crude oil-contaminated soil enhanced marsh plant growth, soil microbial populations and microbial respiration rates, and oil biodegradation rates, indicating the potential for enhancing oil biodegradation. They concluded that bioremediation with fertiliser application significantly reduced total targeted linear and aromatic hydrocarbons remaining in the soil, by 81% and 17%, respectively, compared to those of the non-fertilised controls.

Although it has been shown that the establishment of plants and/or the application of fertiliser can enhance crude oil degradation in soil, plants and degrader-microorganisms may compete for nutrients in soils (Unterbrunner et al. 2007). In order to overcome potential competition for nutrients and/or to overcome nutrient deficiencies, the application of fertilisers may result in enhanced degradation rates of crude oil. Repeated aeration of the contaminated soil may further increase the biodegradation of HC. To test these two hypotheses, we have taken the untreated (i.e., without plants) hydrocarbon-contaminated control soils of the study of Unterbrunner et al. (2007) and investigated the effects of fertilisation with N and P and of agitation on the degradation of crude oil in two incubation experiments.

## MATERIAL AND METHODS

**Experimental soil.** The experimental soils E and G (Ap horizon of Haplic Chernozems) described in Unterbrunner et al. (2007) were obtained from unplanted control pots of a rhizodegradation experiment. The soils had been spiked with crude oil two years before starting the incubation experiments described here (E: 16.4 g/kg; G: 13.3 g/kg). Soil GF was prepared by spiking soil G (13.3 g/kg) again immediately before starting Exp. 2. Before use, the soils were passed through a 2-mm stainless steel mesh. Due to the sieving, initial HC concentrations of soils E and G were higher compared to the values at the end of the experiment presented

in Unterbrunner et al. (2007), where non-sieved soils E and G including the skeleton fraction were used. Extractable N, P and K were determined one month before starting the first incubation experiment. Phosphorus and K were determined in Ca lactate-acetate (CAL) solutions (Schüller 1969),  $N_{\min}$  ( $NH_4^+$  and  $NO_3^-$ ) analysis was performed in 1M KCl (VDLUF, 1991). Phosphorus,  $NH_4^+$  and  $NO_3^-$  were determined using an Agilent 8453 UV VIS spectrophotometer (Agilent Technologies GmbH; Germany) and K was analysed using flame AAS (Perkin Elmer, 2100), respectively. The soil characteristics are shown in Table 1. The concentration of CAL-extractable P was very low in soil E but large in soil G, whereas the concentration of nitrogen was larger in soil G compared to E.

**Fertiliser.** Different amounts (in mg/kg) of N as  $NH_4NO_3$  and P as  $KH_2PO_4$  were applied, resulting in 16 treatments: control, N1 (50), N2 (100), N3 (1000), P1 (25), P2 (50), P3 (500), N1 + P1, N1 + P2, N1 + P3, N2 + P1, N2 + P2, N2 + P3, N3 + P1, N3 + P2, and N3 + P3.

**Incubation of soil.** Experiment 1 was arranged in four replicates. Each incubation glass bottle (200 ml) was filled with 100 g (DW) soil. Soils E and G were incubated for four weeks at 30°C and moistened to 30% of the maximal water holding capacity (MWHC). The incubation was performed two times.

In experiment 2, soils E, G, and GF were subjected to the same incubation procedure. Unfertilised

Table 1. Basic characteristics of the experimental soils E, G and GF

	E	G/GF**
Sand (g/kg)	293	776
Silt (g/kg)	452	164
Clay (g/kg)	254	60
Soil texture	sandy loam	sand
MWHC (g/kg)	638	609
pH (H <sub>2</sub> O)*	8.36	8.39
C <sub>org</sub> (g/kg)*	16.4	19.7
N <sub>tot</sub> (g/kg)*	1.5	1.3
N <sub>min</sub> (mg/kg)	13.9	3.3
P-CAL (mg/kg)	14.4	137
K-CAL (mg/kg)	1.7	2.4
Initial heptane-extractable HC concentration (mg/kg)	3300	2770/4730

\*values obtained from Unterbrunner et al. (2007); \*\*soil GF is identical with soil G but was freshly spiked

controls and selected fertiliser treatments (N3, P3, N3 + P3) were incubated with and without agitation (i.e., manual mixing for one minute three times per week using a glass stirrer).

**Hydrocarbon extraction and analysis.** The extraction and analysis of hydrocarbons was performed as described in Unterbrunner et al. (2007). The detection window solution was prepared as follows: 30 mg *n*-tetracontane (C40) was weighed into 1000 ml volumetric flasks. Then, approximately 500 ml *n*-heptane was added into the flask and closed with a glass cap. The flask was put into the ultrasonic bath until the *n*-tetracontane had dissolved completely. 30 µl (correspond to 21 mg) *n*-decane (C10) was added. Finally, *n*-heptane was added to achieve 1000 ml detection window-solution. Ten g of dry weight of the soil and sand samples were weighed in 100 ml glass bottles with PTFE caps and extracted in a 20 ml 1:1 *n*-heptane/acetone solution (Heptane puriss p.a. ≥ 99.5%, Fluka Chemie GmbH, Germany, acetone puriss p.A. ≥ 99.5%, Fluka Chemie GmbH, Germany) according to DIN ISO16703:2002–2003. Each sample was shaken by hand for 30 s before adding 20 ml of the detection window solution. After shaking for another 30 s, the flask was put in the ultrasonic bath for 60 min. Subsequently the flask was filled up with H<sub>2</sub>O to the same weight and shaken by hand for 30 s. All the samples were centrifuged for 10 min at 2000 rpm. After centrifugation the organic phase (heptane) was separated using a glass pipette and transferred to a 30 ml glass centrifugation vial with a PTFE (polytetrafluorethylene)-cap, adding H<sub>2</sub>O to the same weight. Samples were shaken by hand for 30 s and centrifuged for 5 min at 2000 rpm. The organic phase (heptane) was taken off with a glass pipette and transferred to a 30 ml glass centrifugation vial before adding 0.5 dry Na<sub>2</sub>SO<sub>4</sub> (Fluka Chemie GmbH, Germany) and dry Florisil (activated Mg silicate, Fluka Chemie GmbH, Germany), followed with shaking by hand for 30 s. All samples (clear extracts) were transferred to GC-vials using a glass pipette.

Hydrocarbon analysis was performed by gas chromatography (GC). The used detection window was between C10 to C40 (adapted from ISO 16706:2004). Detection of heptane-extractable, nonpolar hydrocarbons was performed by computerised gas chromatography (Software: HP Chemstation®) equipped with a flame ionisation detector (GC-FID Modell HP 5890 series II; Agilent Technologies, Germany). Sample separation was performed by a 30-m capillary column (123-5731 DB-5HT; 0.32 mm i.d. × 0.1 µm film; Agilent Technologies, Germany) and He was used

as carrier gas. Aliquots of 1.5 µl were injected in split/splitless mode with an injector temperature of 350°C. Column starting temperature of 40°C was held for 4 min and was increased by 20°C/min with a target temperature of 360°C which was held for 10 min. Prior to each sample injection, a tetracontane (C40)-in-heptane standard solution was injected, resulting in chromatograms including hexacontane peaks to be used as an internal C40 standard. To prevent the C40 standard from precipitation at room temperature, tray temperature was adjusted to 50°C by pumping heated water through tray wells. Calibrations were performed using BAM CRM 5004 diesel/grease (1:1 w/w) standard solution (Bundesanstalt für Materialforschung und-prüfung, Germany).

**Analysis of substrate-induced respiration.** Microbial respiration was assessed by substrate induced respiration (SIR). The moist soils were stored at 4°C in aerated bags until further processing. Two g of soil (DW) were weighed into 20-ml glass syringes and treated with 0.5 ml 0.02M glucose solution. The microbial respiration was determined by analysing the evolved CO<sub>2</sub> before and after incubation of the sample (at 25°C for 3–4 h) using gas chromatography (GC 1-5890 Series II Chromatograph). Prior to measurements, the GC was calibrated with 2% ultra pure standard CO<sub>2</sub> gas. Microbial respiration was calculated by measuring peak heights.

**Statistics.** Each treatment was carried out in four replicates. One-factorial ANOVA was carried out to detect significant differences between control and fertilised treatments of Exp. 1 ( $P < 0.05$ ; LSD-test). In Exp. 2, significant differences between agitated and non-agitated treatments were determined using a paired *t*-test ( $P < 0.05$ ).

## RESULTS

**Hydrocarbon incubation experiment 1 (without agitation).** Compared to the control, the HC concentration in soil E was significantly affected only by two fertiliser treatments (N1, N3 + P3) (Figure 1A). Hydrocarbon degradation was inhibited with increasing N fertilisation in the treatments N1, N2, N3. In soil G, fertilisation did not enhance the HC degradation in any treatment (Figure 1B). Similarly, increasing P application inhibited the HC degradation in treatments P1, P2 and P3 (Figure 1 B). Hydrocarbon degradation in soil G was also inhibited in some other fertiliser treatments (Figure 1B).

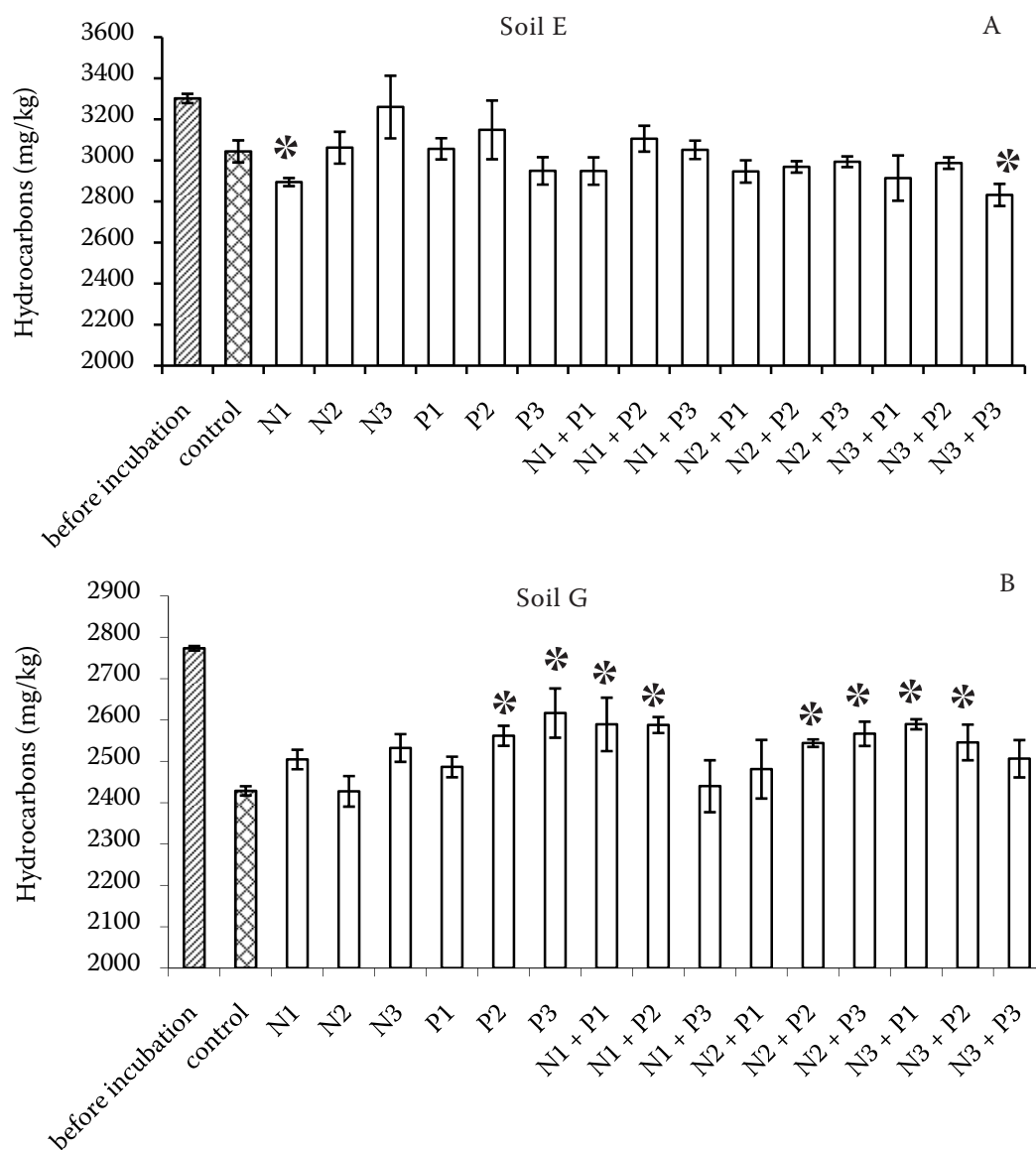


Figure 1. Effect of different N and P fertiliser treatments on HC concentration in soils E and G. Error bars indicate standard errors of the mean,  $n = 4$ . Significant differences ( $P < 0.05$ ; LSD) of fertilised treatments compared to the control are indicated by asterisks

**Hydrocarbon incubation experiment 2 (agitation).** In soil E, non-agitated and fertilised treatments were not significantly different compared to the non-fertilised control (Figure 2). However, the HC concentrations in soils were significantly lower upon aeration due to agitation. Aeration caused better degradation of hydrocarbons in soil, independent of the fertiliser application. The degradation of HC in aged soil G was not affected by agitation, independent of the fertiliser treatments (Figure 2). In contrast, the degradation of HC in fresh soil GF was enhanced by aeration, but not by the fertilisation (Figure 2). Microbial respiration in soil E, G and GF is presented in Figure 3. Microbial respiration rates in soil E were not different between fertilised and non-fertilised

treatments. Microbial respiration in soil G was significantly affected by P fertilisation, but not by agitation. In contrast, P fertilisation did not induce enhanced microbial respiration rates in freshly spiked soil GF. Agitation did not significantly enhance substrate-induced respiration in any fertilised treatments.

## DISCUSSION

Changes of hydrocarbon concentrations during 28 days of incubation at 30°C and 30% MWHC were generally small in Exp. 1 (Figure 1). Biodegradation in both experimental soils (E, G) is likely limited by low bio-accessibility of hydrocarbons as a con-

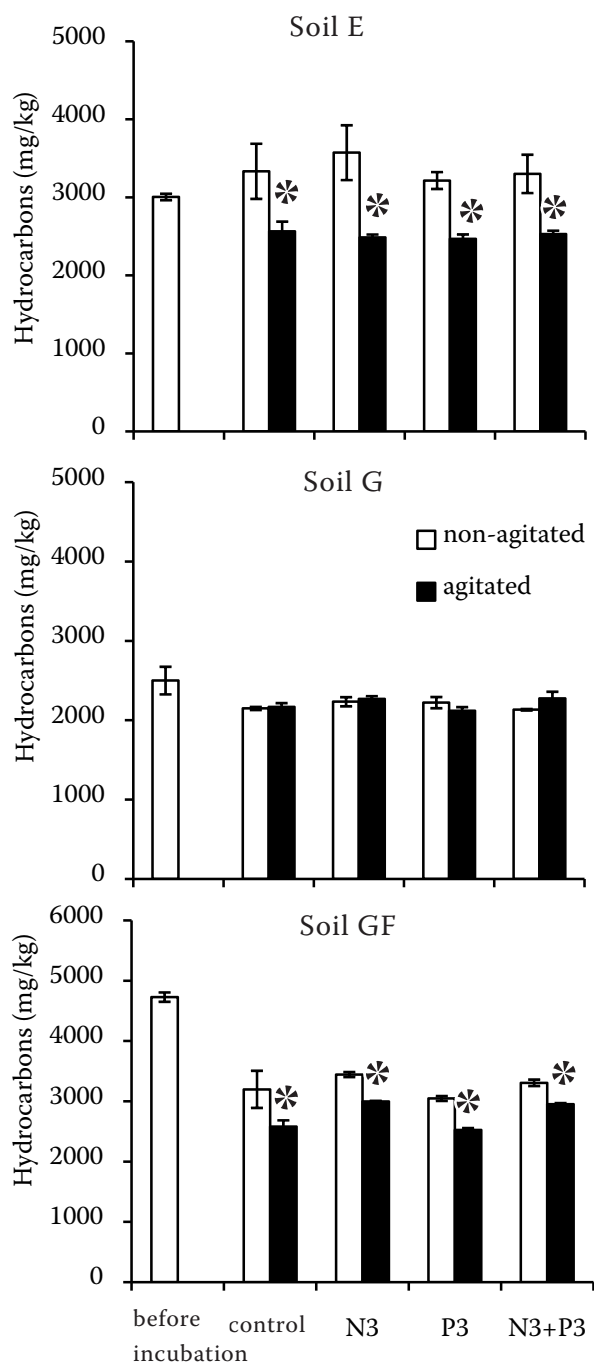


Figure 2. Effect of N and/or P fertilisation and agitation on HC concentration in soils E, G and GF. Error bars indicate standard errors of the mean,  $n = 4$ . Significant differences ( $P < 0.05$ ; LSD) between agitated and non-agitated treatments are indicated by asterisks

sequence of long-term ageing (2 years). Further limitations of biodegradation may include low bioavailability of N (soil G; less pronounced in soil E) and P (soil E). Based on the soil textural classification, there is an indication for limited oxygen supply in soil E as the high proportion of clay and silt fractions limits gas diffusion, in par-

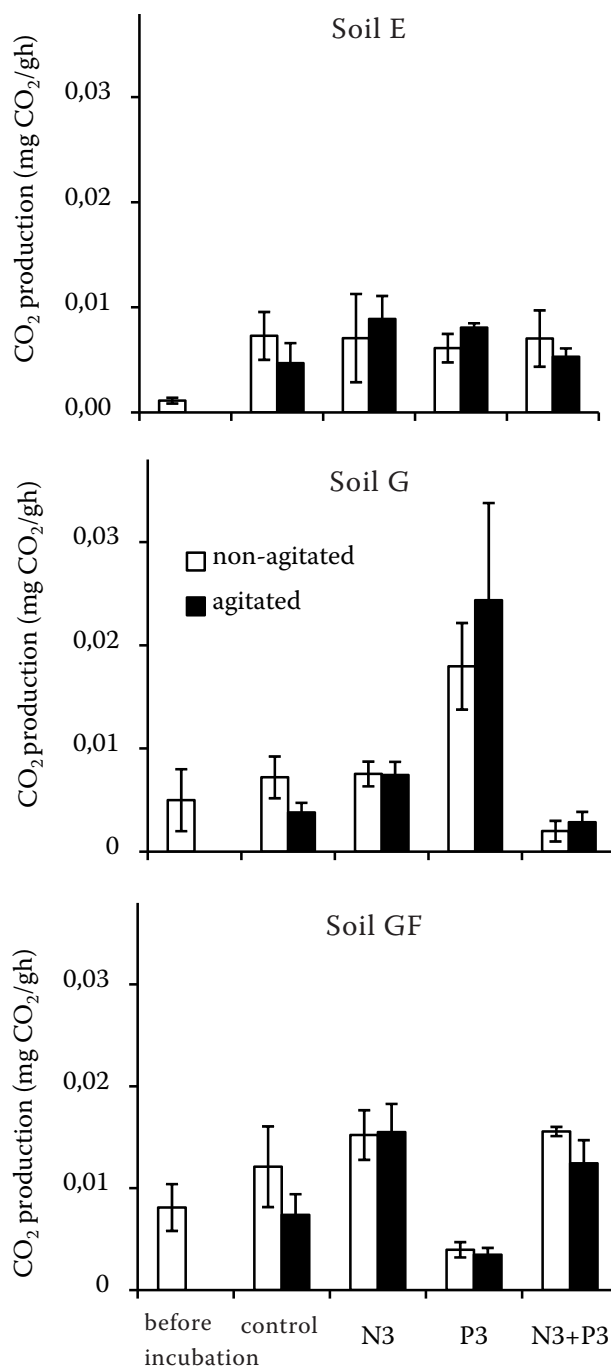


Figure 3. Effect of N and/or P fertilisation and agitation on substrate-induced respiration rates (SIR) of soils E, G and GF. Error bars indicate standard errors of the mean,  $n = 4$ . No significant differences ( $P < 0.05$ ; LSD) were found between agitated and non-agitated treatments

ticular in the specific experimental conditions, i.e. the incubation of sieved ( $< 2$  mm), homogenized soil lacking structure.

Fertilisation with N, P or their combinations was ineffective in most treatments of Exp. 1. Marginally enhanced but significant HC degradation, compared to the control, could only be detected for



the lowest N (N1) and the highest N + P (N3 + P3) application to soil E. In soil G, most fertilizer treatments slightly inhibited HC degradation compared to the control. HC degradation was increasingly inhibited upon increasing N application (N1 → N3) to soil E which had high initial N concentration (Table 1). Similarly, HC degradation decreased with increasing P fertilisation in soil G which was high in initial P (Table 1). Reduction of crude oil degradation rates due to excess fertilisation were also reported by Walworth et al. (1997, 2007). The absence of positive N and P fertilizer effects on HC degradation in most treatments of soil E and the inhibition in soil G further suggests that other factors (bioavailability of HCs, oxygen supply) than N and P nutrition were limiting, even though the availability of both nutrients was lower than in the initial soils used for the previous rhizodegradation experiment (Unterbrunner et al. 2007).

Experiment 2 included agitation to improve oxygen supply in the experimental soils in the presence or absence of fertilization (Figures 2 and 3). Confirming results of Exp. 1, fertilisation had no significant effects on HC biodegradation. However, agitation enhanced HC biodegradation in soil E by about 15% compared to non-agitated treatments. In contrast, we observed no effect of agitation in soil G. This difference can be explained by a larger remaining biodegradable HC fraction after 2 years of biodegradation and ageing and limited oxygen supply in the non-agitated treatments of this soil as a consequence of its heavier texture. In contrast, biodegradation was more effective in soil G because of better bio-accessibility and oxygen supply in this sandy soil. Improved oxygen supply through agitation therefore could not enhance HC degradation but was effective in the same soil, freshly HC-spiked (soil GF).

Successful degradation of HC in soils and sediments depends on managing the behaviour of contaminants and the environmental conditions. Usually, contaminated soils that have aged for long periods may be less susceptible for microbial-induced HC degradation.

Bioaccessibility of the hydrocarbon pollutants is critical for successful degradation by microbes. Although Unterbrunner et al. (2007) showed that availability of nutrients (especially P) was critical in their rhizodegradation experiment, fertilisation did not enhance biodegradation rates in the subsequent incubation study presented here due to the low availability of the pollutants. Enhancement of oxygen supply was only effective in the freshly spiked soils GF and the heavy-textural soil E. Our

results indicate very limited applicability of biodegradation to remediation of HC-contaminated soils after extended period of ageing. Most studies showing efficient biodegradation were conducted using freshly spiked soil, which has limited implication for the real field situation.

## REFERENCES

- Atlas R.M. (1995): Bioremediation of petroleum pollutants. *International Biodeterioration Biodegradation*, 35: 317–327.
- Boethling R.S., Alexander M. (1979): Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. *Applied and Environmental Microbiology*, 37: 1211–1216.
- Bragg J.R., Prince R.C., Harner E.J., Atlas R.M. (1994): Effectiveness of bioremediation for the Exxon Valdes oil spill. *Nature*, 368: 413–418.
- Cleveland C.J. (2007): Hydrocarbon Chemistry. In: Cleveland C.J.: *Encyclopedia of Earth*. Environmental Information Coalition, National Council for Science and the Environment Washington, D.C. First published in the *Encyclopedia of Earth* August 30, 2006; Last revised February 4, 2007; Retrieved June 8, 2009. Available at: [http://www.eoearth.org/article/Hydrocarbon\\_chemistry](http://www.eoearth.org/article/Hydrocarbon_chemistry)
- Killops S.D., Al-Jaboory M.A.H.A. (1990): Characterisation of the unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleum. *Organic Geochemistry*, 15: 147–160.
- Lin Q., Mendelsson I.A., Henry C.B., Roberts P.O., Walsh M.M., Overton E.B., Portier R.J. (1999): Effects of bioremediation agents on oil degradation in mineral and sandy salt marsh sediments. *Environmental Technology*, 20: 825–837.
- Margesin R., Zimmerbauer A., Schinner F. (2000): Monitoring of bioremediation by biological activities. *Chemosphere*, 40: 339–346.
- Oudot J., Merlin F.X., Pinvidic P. (1998): Weathering rates of oil components in a biodegradation experiment in estuarine sediments. *Marine Environmental Research*, 45: 113–125.
- Pritchard P.H., Costa C.F. (1991): EPA's Alaska oil spill bioremediation project. *Environmental Science and Technology*, 25: 372–379.
- Schüller H. (1969): Die CAL-Methode, eine neue Methode zur Bestimmung des pflanzenverfügbaren Phosphates in Böden. *Zeitschrift für Pflanzenernährung und Bodenkunde*, 123: 48–63.
- Unterbrunner R., Wieshammer G., Hollender U., Felderer B., Wieshammer-Zivkovic M.W., Puschenreiter M., Wenzel W.W. (2007): Plant and fertiliser effects on rhizodegradation of crude oil in two soils with different nutrient status. *Plant and Soil*, 300: 117–126.
- Van Hamme J.D., Singh A., Ward O.P. (2003): Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews*, 67: 503–549.

VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten) (1991): Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik, Methodenbuch Band 1: Die Untersuchung von Böden. VDLUFA-Verlag, Darmstadt.

Walworth J.L., Woolard C.R., Braddock J.F., Reynolds C.M. (1997): Enhancement and inhibition of soil petroleum biodegradation through the use of fertilizer nitrogen: an approach to determining optimum levels. *Journal of Soil Contamination*, 6: 465–480.

Walworth J.L., Pond A., Snape I., Rayner J., Ferguson S., Harvey P. (2007): Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. *Cold Regions Science and Technology*, 48: 84–91.

Wright A.L., Weaver R.W., Webb J.W. (1997): Oil bioremediation in salt marsh mesocosms as influenced by N and P fertilization, flooding and season. *Water, Air, and Soil Pollution*, 95: 179–191.

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