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# A feasibility study on the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site: Effects of temperature and biostimulation

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ABSTRACT

A laboratory feasibility study on the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site was conducted over a period of 30 weeks. We determined the effects of temperature (10 °C and 20 °C) and of various biostimulation treatments (inorganic nitrogen–phosphorus–potassium fertilization and the two commercial products Inipol EAP22 and Terramend) versus natural attenuation on the loss of total petroleum hydrocarbons (TPH), microbial activity (soil respiration) and community composition (phospholipid fatty acids, PLFA). The hydrocarbon contamination was removed almost completely (up to 92.7%) at 20 °C, while at 10 °C losses up to 69% were obtained. Biostimulation by the addition of nutrients had a significantly stimulating effect on the biodegradation activity of the indigenous soil microorganisms; however, a considerable amount of hydrocarbon loss could be attributed to natural attenuation. Shifts in microbial community composition during bioremediation included the significant increase of soil fungi at 10 °C and of Gram-negative soil bacteria at 20 °C. Significantly positive correlations between hydrocarbon loss, soil respiration and patterns of phospholipid fatty acids demonstrated the involvement of a wide range of soil microorganisms (Gram-positive and Gram-negative bacteria, fungi) in the bioremediation of the investigated soil.



## 1. Introduction

Petroleum hydrocarbons are the most widespread contaminants in the environment. A number of methods, including physical, chemical and biological treatments, are available for the treatment of petroleum contamination (Ma and Jiao, 2012; Singh and Ward, 2004; van Hamme et al., 2003). Contaminants can be degraded or otherwise be made harmless by natural processes (natural attenuation) that include biodegradation by indigenous microorganisms and a number of abiotic processes, such as dispersion, sorption and transformations (Hinchee, 1998). Bioremediation attempts to accelerate the natural biodegradation rates through the optimization of limiting environmental conditions and is an ecologically and economically effective method. The basis for the development of biological remediation methods is the capacity of a broad spectrum of microorganisms to utilize hydrocarbons as the sole source of carbon and energy (biodegradation) and has been described already very early (ZoBell, 1946). The most widely used bioremediation procedure in cold soils is biostimulation of the indigenous microorganisms by supplementation of appropriate nutrients and optimization of other limiting factors, such as oxygen content, pH and temperature control. Bioaugmentation by inoculating allochthonous hydrocarbon degraders has been used as a bioremediation option to treat petroleum-contaminated cold and temperate sites. However, this strategy generally underperformed or gave no better results than fertilization (Filler et al., 2009; Margesin, 2004; Tyagi et al., 2011).

Cold environments are increasingly exposed to petroleum exploration, production and transport, and these activities increase the risk of accidental oil release. Such environments include polar and alpine soils, permafrost, sediments, sea, and also subsoils and groundwaters of temperate climates where temperatures rarely exceed 10 °C. In soils, deep penetration of hydrocarbons from the topsoils into the subsoils represents a direct risk of groundwater contamination. Successful bioremediation of hydrocarbon-contaminated soils in various cold environments, including the Arctic, Antarctic and Alpine areas, has been reported (Bej et al., 2010; Brakstad et al., 2008; Filler et al., 2008; Greer et al., 2010). Several remediation schemes, such as biopiles, landfarming and engineered bioremediation have been implemented successfully at petroleum-contaminated cold sites (e.g. Filler et al., 2006, 2009; Paudyn et al., 2008; Walworth and Ferguson, 2008).

To evaluate the performance of microorganisms during hydrocarbon bioremediation, monitoring methods are applied. Soil biological activities, e.g. soil respiration and enzyme activities, give information on the impact of the effects of environmental stress, such as contamination, on soil metabolic activity (Chakraborty et al., 2012; Margesin and Schinner, 2005). Direct, non-culture-based, molecular methods, e.g. soil DNA or phospholipid fatty acid (PLFA) profiling, provide information on the microbial community structure (Chakraborty et al., 2012; Macnaughton et al., 1999; Zelles, 1999). Specific PLFA patterns reflect the physiological stress, the nutritional status and the viable

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biomass of the microbial population (Macnaughton et al., 1999; Schinner et al., 1996; Zelles, 1999).

It was the objective of this study to investigate the feasibility of bioremediation of petroleum–hydrocarbon-contaminated soil collected from an Alpine former military site. In a laboratory study, we investigated the natural attenuation and determined the effect of biostimulation by applying three different fertilizers. Changes in the hydrocarbon concentration, microbial activity (respiration) and community composition (PLFA patterns) were monitored over a period of 30 weeks at 10 °C and at 20 °C. These two temperatures correspond to the mean annual soil temperature in the sampling area (10 °C) and to the temperature that the soil would be manipulated to as part of the bioremediation treatment (20 °C).

## 2. Materials and methods

### 2.1. Sampling site and soil sampling

Soil samples were collected from a former military site in October 2010. The site was located in the European Alpine region in Welsberg-Taisten (46° 45′ 10.57″ N; 12° 06′ 47.39″ E), South Tyrol, Italy. The area used to be a military site until 1990 . It had a size of 20,000 $m^2$ and hosted 21 buildings (caserns, storage rooms, shooting stand, garages, etc.). After the removal of 11 underground storage tanks for heating oil, diesel oil and gasoline, a preliminary examination of soil samples pointed to petroleum hydrocarbon contamination. Since the area is within a drinking water protection area and thus in an especially environmentally sensitive area, the authorities decided to examine the feasibility of bioremediation treatment. The contaminated soil material was excavated with the help of a bucket to a depth of 3–5 m deep. Five soil samples (15 kg each) of the excavated material were collected and immediately transported to the laboratory. All soil samples were gently crumbled and sieved through a 6.3 mm screen in order to eliminate rough materials, thoroughly mixed, and stored at field humidity at 4 °C until processing. Immediately before the setup of the bioremediation experiment, a composite sample of the five collected samples was produced.

### 2.2. Biostimulation experiment

Eight 2-L pans containing 1.5 kg (fresh mass) of the composite soil sample were prepared. To test the effect of four treatments,

(1) Two pans remained unfertilized to evaluate natural attenuation.
(2) Two pans were amended with an agricultural inorganic NPK fertilizer (containing 9.5% $NH_3$-N, 5.5% $NO_3$-N, 6.6% $P_2O_5$-P and 12.2% $K_2O$-K; N:P = 2.3:1, K:P = 1.8:1) using a C:N ratio of 20:1 for the initial hydrocarbon concentration. Pre-investigations on the effect of various C:N ratios (10:1, 20:1, 30:1, 40:1; 50:1) showed that a C:N ratio of 20:1 was optimal for the bioremediation treatment of the studied soil (data not shown). The N concentration in soil solution as calculated according to Walworth et al. (2007) was 2230 mg N $kg^{-1}$ soil $H_2O$, which is in agreement with the recommendation by Walworth et al. (2007), according to which microbial activity is reduced at $N_{H_2O}$ above 2500 mg N $kg^{-1}$ soil $H_2O$.
(3) Two pans were amended with the oleophilic fertilizer Inipol EAP22® (Elf Atochem North America, Inc., Philadelphia) using hydrocarbons:EAP ratio of 10:1 (w/w) as recommended (Bruchon et al., 1996). The product is an oil-in-water emulsion containing organic nutrients: N in the form of urea emulsified with oleic acid, P in the form of lauryl phosphate, and trace quantities of an emulsion stabilizer (Churchill et al., 1995). Information on the N:P content of this fertilizer is contradictory and ranges from 2.6:1 (Pritchard et al., 1992) to 18.5:1, as calculated from a C:N:P ratio of 62:7.4:0.7 (Coulon et al., 2005).
(4) Two pans were amended with the commercial product Terramend® hydrocarbons (FMC Environmental Solutions) at a final concentration of 1% (w/w) as recommended by the manufacturer for initial TPH (total petroleum hydrocarbons) contents of 4000–10,000 mg $kg^{-1}$ soil dry mass. This product consists of major, minor and micronutrients (35–45% monoammonium phosphate, 20–25% calcium carbonate, 0–2% ammonium nitrate, 0–2% monobasic ammonium phosphate, 0–2% potassium nitrate, 20–30% organic amendment not further specified).

The soil water content was adjusted in all pans with sterile water to ca. 50% of the maximum water holding capacity. The pans were closed with covers that contained six holes (diameter 2 cm) closed with cotton wool stoppers. One pan per treatment was incubated in the dark at 20 °C and 50% relative humidity, while the second pan of each treatment was incubated at 10 °C. The soil in the pans was mixed thoroughly twice a week, whereby water loss during incubation was compensated for by the addition of sterile water. Immediately after the setup of the experiment, weekly (up to 8 weeks) and afterwards in longer time intervals (10, 12, 15, 20, 25 and 30 weeks), soil samples (ca. 50 g) were removed from each pan to determine the residual TPH concentration, soil dry mass and soil pH. After 0, 15 and 30 weeks, ca. 20 g of soil samples was collected for PLFA analysis.

### 2.3. Physical and chemical soil properties

Physical and chemical soil properties were examined with 3 replicates. Soil dry mass content was determined from mass loss after 24 h at 105 °C. Soil pH was determined in 10 mM $CaCl_2$. Soil nutrient contents (nitrate, nitrite, phosphorus) of the soil sample at the beginning of the experiment were determined spectrophotometrically (Schinner et al., 1996).

Hydrocarbon content in the range of $C_{10}$ to $C_{40}$ was determined by gas chromatography and a flame ionization detector after extraction (three replicates) with heptane (DIN ISO 16703, modified according to ÖNORM EN 14309).

### 2.4. Soil respiration

The setup for the measurement of soil respiration occurred simultaneously with the setup of the decontamination experiment. Soil respiration was determined by using the system OxiTop® Control (WTW). Twenty grams of soil fresh mass was weighed into each of 24 100-ml vessels. Six vessels each were treated as described for the biostimulation experiment (see Section 2.2). The vessels were provided with the corresponding measuring heads that were filled with NaOH platelets. Afterwards the vessels were tightly closed and three vessels per treatment were incubated at 10 °C and at 20 °C. The consumption of oxygen due to soil respiration resulted in a negative pressure that was constantly monitored via the OxiTop® Control measuring heads. NaOH platelets were regularly replaced.

### 2.5. PLFA

Phospholipids were determined with three replicates as previously described (Margesin et al., 2007, 2009) and were extracted, fractionated and quantified using the procedures described (Bardgett et al., 1996; Frostegard et al., 1993). Separated fatty acid methyl-esters were identified using gas chromatography and a flame ionization detector. Fatty acid nomenclature was used as described (Frostegard et al., 1993). The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω7c and cy19:0 were chosen to represent bacterial biomass (bacterial PLFA), and 18:2ω6,9c (fungal PLFA) was taken to indicate fungal biomass (Zelles, 1999). The Gram-positive specific fatty acids i15:0, a15:0, i16:0 and i17:0 and the Gram-negative specific fatty