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Efficiency of Indigenous and Inoculated Cold-Adapted Soil Microorganisms for Biodegradation of Diesel Oil in Alpine Soils

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Biodegradation of diesel oil (5 g · kg [soil dry weight]$^{-1}$) was investigated in five alpine subsoils, differing in soil type and bedrock, in laboratory experiments during 20 days at 10°C. The biodegradation activities of the indigenous soil microorganisms and of a psychrotrophic diesel oil-degrading inoculum and the effect of biostimulation by inorganic fertilization (C/N/P ratio = 100:10:2) were determined. Fertilization significantly enhanced diesel oil biodegradation activity of the indigenous soil microorganisms. Biostimulation by fertilization enhanced diesel oil biodegradation to a significantly greater degree than bioaugmentation with the psychrotrophic inoculum. In none of the five soils did fertilization plus inoculation result in a higher decontamination than fertilization alone. A total of 16 to 23% of the added diesel oil contamination was lost by abiotic processes. Total decontamination without and with fertilization was in the range of 16 to 31 and 27 to 53%, respectively.

The microbiological decontamination (bioremediation) of oil-polluted soils is claimed to be an efficient, economic, and versatile alternative to physicochemical treatment (1, 3). Bioremediation involves the use of indigenous or introduced (allochthonous) microorganisms to detoxify and degrade environmental contaminants. The rate of microbial degradation of hydrocarbons in soils is affected by several physicochemical and biological parameters including the number and species of microorganisms present; the conditions for microbial degradation activity (e.g., presence of nutrients, oxygen, pH, and temperature); the quality, quantity, and bioavailability of the contaminants; and the soil characteristics (e.g., particle size distribution).

The optimum temperature for biodegradation of mineral oil hydrocarbons in temperate climates has generally been found to be in the range of 20 to 30°C (2). Local environmental conditions like cold climates may select for populations with a lower optimum temperature: ZoBell (29) reported mineral oil degradation at temperatures below 0°C; after an oil spill in Alaska, high biodegradation activities of indigenous soil microorganisms were observed at 10 to 16°C (18).

In alpine soils, temperatures above 10°C are reached only during the period of high solar irradiation and on hot summer days. In subsoils, temperatures around 8 to 10°C prevail. These conditions greatly influence soil microbial conversion and degradation rates and demand a high level of microbial cold adaptation. Cold-adapted, psychrophilic and psychrotrophic, microorganisms are distinguished from mesophiles (whose optimum growth temperature is between 20 and 40°C) by their ability to grow and multiply at 0°C. Psychrophiles and psychrotrophs are distinguished by their minimum, optimum, and maximum growth temperatures; these are <0, <15, and <20°C, respectively, for psychrophiles and 0 to 5, >15, and >20°C, respectively, for psychrotrophs (17). The metabolism of cold-adapted microorganisms is adapted to work and function optimally at low temperatures (14, 20); such organisms could be useful for bioremediation processes in cold environments like the alpine and arctic areas (9, 14, 27).

There is no information available on oil degradation in the alpine environment as yet, although oil pollution is likely to occur with leaks, storage tank ruptures, and transport accidents on alpine roads and in ski resorts. We evaluated the biodegradation of diesel oil at a low temperature (10°C) in five alpine subsoils in a laboratory study. The biodegradation activity of the indigenous soil microorganisms, the efficiency of bioaugmentation by addition of a psychrotrophic diesel oil-degrading inoculum, the effect of biostimulation by inorganic fertilization, and the role of abiotic losses were determined.

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MATERIALS AND METHODS

Soils. The soils selected for this study were originally uncontaminated subsoils from five areas in the Tyrolean Alps in Austria (Table 1). Soil samples were taken from C horizons, and processing began immediately upon arrival at the laboratory. Soil samples were sieved moist through a 5-mm-mesh screen and thoroughly mixed. Investigations started immediately after sieving. Physicochemical properties (as presented in Table 2) were determined as described earlier (21).

We determined soil microbial counts by the most-probable-number (MPN) technique. Uncontaminated soil material corresponding to 10 g (dry weight) was incubated for 30 min with 90 ml of sterile pyrophosphate (2.8 g liter^{-1}) and 2 drops of Tween 80 at 10°C and 150 rpm. One milliliter of appropriate dilutions of the soil suspension was inoculated into 9 ml of a pH-neutral phosphate-buffered mineral medium (6), containing $(NH_4)_2SO_4$ (1 g liter^{-1}) as the nitrogen source. For determination of diesel oil utilizers, we added 20 μl of diesel oil (1.64 g liter^{-1}). We also determined the MPN of a subset of heterotrophic microorganisms that grow in the same mineral medium with glucose (2 g liter^{-1}) as the sole carbon source. Three-tube MPN determinations were used. The tubes were incubated at 10°C and 150 rpm; visual appearance of growth after 10 to 14 days was used to score positive tubes.

Psychrotrophic inoculum. The investigated strain was isolated from a diesel oil-contaminated alpine soil and selected from among 144 isolates because of its high diesel oil biodegradation activity at 10°C. On the basis of morphological and physiological features, the isolate was identified as *Yarrowia lipolytica* (Wickerham et al.) van der Walt and von Arx (25, 28). The strain exhibited the properties of a psychrotroph (17), showing a growth temperature range of 0 to 30°C and an optimum growth temperature of 15 to 20°C.

Preparation of inoculum. Cultivation was done in 250-ml Erlenmeyer flasks at 10°C and 180 rpm in a phosphate-buffered pH-neutral mineral salt medium (6), containing $(NH_4)_2SO_4$ (1 g liter^{-1}) as the source of nitrogen and diesel oil (5 g

TABLE 1. Description of soil sampling locations[a]

Soil no.	Location (Austria)	Ht (m) above sea level	Bedrock	Soil type
1	Kühtai	2,000	Silicate	Alpine pseudogley
2	Hahntennjoch	1,715	Ca-carbonate	Brunificated pararendzina
3	Zwieselstein, Ötztal	1,470	Silicate	Ranker, brown earth
4	Gnadenwald	880	Ca-carbonate	Alpine moder rendzina
5	Innsbruck	560	Ca-carbonate, silicate	Pararendzina

[a] For all soils, the horizon was C (subsoil).

liter^{-1}) as the sole source of carbon and supplemented with yeast extract (10 mg liter^{-1}). When the culture reached the late logarithmic phase and showed a high biodegradation activity (after 10 days, 68% of the diesel oil was biodegraded by the inoculum), cells were centrifuged, washed twice with a sterile 0.9% NaCl solution, and suspended in 0.9% NaCl solution.

Decontamination experiments. Sixty 100-ml Erlenmeyer flasks were prepared for each of the soils investigated. Each flask contained 25 g of soil, which was contaminated and mixed with 5 g of diesel oil · kg (soil dry weight)$^{-1}$ (85.7% C; density, 820 g liter^{-1}). We tested five treatments per soil. For the determination of abiotic hydrocarbon losses, poisoned controls (12 flasks per soil) contained 0.3% (wt/wt) $AgNO_3$. To assay the effect of indigenous soil microorganisms, 12 flasks per soil received no treatment, i.e., neither inoculation nor fertilization. For assaying the effect of bioaugmentation, 12 flasks per soil were amended with 5% inoculum (10^8 CFU g [dry weight]$^{-1}$). To determine the effect of biostimulation (fertilization), 12 flasks per soil received an agricultural, water-soluble inorganic fertilizer (N/P ratio = 5:1; P/K ratio = 0.5:1; Agrolinz Melamin GmbH, Linz, Austria); the C/N ratio was adjusted to 10:1. For assaying the effect of biostimulation plus bioaugmentation, 12 flasks per soil received both the fertilizer (C/N ratio = 10:1) and the inoculum (10^8 CFU g [dry weight]$^{-1}$).

The water content was adjusted with sterile water to 60% of the maximum water holding capacity in all flasks. Water losses during incubation were compensated for regularly by the addition of sterile water. The flasks were closed with cotton wool stoppers and incubated in the dark at 10°C for 20 days. In order to avoid anaerobic conditions, the contents of the flasks were mixed thoroughly every second day.

Mineral hydrocarbon content. After 5, 10, 15, and 20 days, we removed three flasks of each of the five treatments to measure the residual total petroleum hydrocarbon concentration according to the German standard method DIN 38 409-H18 (4). The soil was mixed and dehydrated with water-free Na_2SO_4. After extraction for 30 min with 30 to 40 ml of 1,1,2-trichloro-trifluoro-ethane by head-over-head rotation, the CH_3 and CH_2 groups of the diesel oil were quantitatively determined by infrared spectroscopy.

In order to determine the diesel oil recovery from the investigated soils (t = 0 values), we prepared three flasks for each soil. Each flask contained 25 g of soil and diesel oil at a concentration of 5 g · kg (dry weight)$^{-1}$. The mineral hydrocarbon content was determined immediately afterwards.

Statistical data analyses. Data were found to be normally distributed according to the Kolmogorov-Smirnow test. Data were analyzed by analysis of variance ($P \leq 0.05$) and by multiple range analysis (least significant difference [LSD], $P \leq 0.05$). Treatments were considered significant when P was $\leq$0.05.

RESULTS

Soil analyses. Physical and chemical properties of the investigated soils are presented in Table 2. The native soil hydrocarbon contents in the investigated soils were below 0.1 g · kg (dry weight)$^{-1}$ and could therefore be neglected, as this amount did not interfere with diesel oil bioremediation experiments.

The numbers of glucose-utilizing microorganisms of the investigated soils before contamination were in the range of 6×10^5 (soil 3) and 2×10^7 (soil 2) g (dry weight)$^{-1}$ (Table 3). In soils 1, 2, and 3, a significant part of these populations consisted of microorganisms that were able to utilize diesel oil as the sole source of carbon and energy (2×10^4 to 4×10^4 g [dry weight]$^{-1}$). Soils 4 and 5 contained 2×10^3 diesel oil utilizers g (dry weight)$^{-1}$ (Table 3).

Diesel oil recovery. The recovery yields of added substances are known to be greatly influenced by soil characteristics (21). We were able to recover 93 to 95% of the added diesel oil (5 g · kg [dry weight]$^{-1}$) from the investigated soils immediately after contamination (t = 0), (Table 4).

Abiotic hydrocarbon losses. With the poisoned controls, in which indigenous soil microorganisms were inhibited by silver ions, we recorded the diesel oil losses due to abiotic processes. In preliminary experiments, 0.3% $AgNO_3$ was estimated to be sufficient to inhibit soil microorganisms completely; in all poisoned controls, the number of soil microorganisms was $<10^2$ g (soil dry weight)$^{-1}$.

After 20 days at 10°C, 16 to 23% of the added diesel oil contamination, corresponding to 0.8 to 1.15 g of hydrocarbons · kg (dry weight)$^{-1}$, was eliminated by abiotic processes (Table 4). With all five soils, abiotic losses were not, or only to a marginal extent, influenced by the presence of fertilizer (data not shown). Abiotic hydrocarbon loss was statistically significantly ($P \leq 0.05$) higher in soil 3 than in soil 4; abiotic losses in soils 1, 2, and 5 were not significantly different (Table 5).

Diesel oil biodegradation activities in unfertilized soils. In untreated soils (without biostimulation and without bioaugmentation [−F −I]), diesel oil biodegradation activity of the indigenous soil microorganisms was negligible in all five soils. Except for soil 5, the total hydrocarbon loss (16 to 25% after 20 days) could almost entirely be attributed to abiotic processes (Tables 4 and 5).

The addition of a psychrotrophic inoculum to the unfertil-

TABLE 2. Properties of the investigated soils

Soil no.	pH[a]	Total N (%)	Humus (%)	Organic C (%)	Inorganic C (%)	$CaCO_3$ (%)	Hydrocarbons[b] (mg · kg [dry wt]$^{-1}$)	P_2O_5 (mg · kg [dry wt]$^{-1}$)	K_2O (mg · kg [dry wt]$^{-1}$)	Particle size (%)[c]		
										Sand	Silt	Clay
1	6.2	<0.2	0.6	0.3	<0.01	<0.1	7	20	30	84	13	3
2	7.2	0.09	2.7	1.4	11.6	97	21	20	10	53	45	2
3	6.5	<0.02	0.9	0.5	<0.01	<0.1	15	40	70	86	12	2
4	5.0	0.09	3.0	1.7	<0.01	<0.1	81	<10	30	41	49	10
5	7.5	0.09	2.6	1.6	4.9	41	97	90	80	58	36	6

[a] Measured in 10 mM $CaCl_2$.
[b] Native soil hydrocarbons.
[c] Sand plus silt plus clay = 100%.

TABLE 3. Numbers of diesel oil utilizers and glucose utilizers in the investigated soils before contamination, as determined by the MPN technique ($n = 3$)

Soil no.	No./g (dry wt) of soil	
	Diesel oil utilizer	Glucose utilizer
1	$(3.2 \pm 2.9) \times 10^4$	$(1.7 \pm 1.6) \times 10^6$
2	$(3.7 \pm 3.3) \times 10^4$	$(1.7 \pm 1.6) \times 10^7$
3	$(1.7 \pm 1.6) \times 10^4$	$(6.1 \pm 5.6) \times 10^5$
4	$(1.5 \pm 1.3) \times 10^3$	$(6.1 \pm 5.6) \times 10^6$
5	$(1.5 \pm 1.3) \times 10^3$	$(1.7 \pm 1.6) \times 10^6$

ized soils (−F +I) resulted in only a small increase (5 to 7%) of the total hydrocarbon loss. A statistically significant difference ($P \leq 0.05$) between inoculated and noninoculated unfertilized soils was observed after 10 to 15 days of incubation in soils 2, 3, and 5 and was noticed after 20 days also in soil 1 (Table 4).

After 20 days at 10°C, total hydrocarbon loss in the unfertilized soils was significantly ($P \leq 0.05$) higher in soils 2, 3, and 5 than in soils 1 and 4; this result was not influenced by the presence or absence of the inoculum (Table 5).

TABLE 4. Effects of fertilization (F) and inoculation (I) on total hydrocarbon loss in five alpine subsoils after 0 to 20 days at 10°C[a]

Soil no.	Time (days)	Total hydrocarbon loss (%)[b]				
		Poisoned control	−F −I	−F +I	+F −I	+F +I
1	0	6.47	6.47	6.47	6.47	6.47
	5	10.87 a	12.97 a	12.98 a	14.71 ab	16.22 ab
	10	12.34 a	12.62 a	14.34 a	21.29 b	23.65 c
	15	14.87 a	16.60 ab	17.28 b	27.00 c	28.32 c
	20	16.40 a	16.43 a	21.05 b	32.58 c	32.32 c
2	0	5.11	5.11	5.11	5.11	5.11
	5	14.77 a	14.51 a	13.64 a	15.21 a	13.53 a
	10	11.87 a	14.97 a	19.60 b	33.79 c	32.79 c
	15	13.55 a	21.64 b	26.64 c	43.19 d	45.33 d
	20	21.27 a	24.93 ab	29.50 b	53.28 c	52.75 c
3	0	3.85	3.85	3.85	3.85	3.85
	5	13.36 a	14.28 a	13.36 a	18.99 ab	21.98 b
	10	15.46 a	15.74 a	24.22 b	21.45 b	28.52 c
	15	17.74 a	17.92 a	26.64 b	26.47 b	33.34 c
	20	22.94 a	23.45 a	30.57 b	33.94 bc	34.73 c
4	0	4.96	4.96	4.96	4.96	4.96
	5	9.53 a	8.79 a	9.09 a	11.78 b	9.54 a
	10	10.92 a	12.09 a	12.87 a	18.34 b	18.69 b
	15	10.26 a	15.24 b	16.31 bc	19.50 c	19.65 c
	20	16.48 a	17.99 ab	21.96 ab	27.29 c	30.94 c
5	0	5.48	5.48	5.48	5.48	5.48
	5	10.63 a	11.68 a	14.96 b	13.54 ab	11.98 ab
	10	11.57 a	17.19 b	21.58 c	26.20 d	26.55 d
	15	13.17 a	21.54 b	24.47 c	30.81 d	30.40 d
	20	15.73 a	23.88 b	29.82 c	38.87 d	37.10 d

[a] The initial contamination was 5 g of diesel oil · kg (dry weight)$^{-1}$ (= 100% hydrocarbon content).

[b] Values represent the means of three replicates; the typical standard error for means was ±15% of mean or less. Different letters (a, b, c, and d) in a row indicate statistically significant differences (LSD, $P \leq 0.05$) between treatments; the same letters indicate that values are not significantly different.

TABLE 5. Effects of fertilization (F) and inoculation (I) on total hydrocarbon loss in five alpine subsoils after 20 days at 10°C[a]

Treatment	Total hydrocarbon loss (%) for soil no.[b]:				
	1	2	3	4	5
Poisoned control	16.40 ab	21.27 ab	22.94 b	16.48 a	15.73 ab
−F −I	16.43 a	24.93 b	23.45 b	17.99 a	23.88 b
−F +I	21.05 a	29.50 b	30.57 b	21.96 a	29.82 b
+F −I	32.58 b	53.28 d	33.94 b	27.29 a	38.87 c
+F +I	32.32 a	52.75 b	34.73 a	30.94 a	37.10 a

[a] The initial contamination was 5 g of diesel oil · kg (dry weight)$^{-1}$ (= 100% hydrocarbon content).

[b] Values represent the means of three replicates; the typical standard error for means was ±15% of mean or less. Different letters (a, b, c, and d) in a row indicate statistically significant differences (LSD, $P \leq 0.05$) between soils; the same letters indicate that values are not significantly different.

Diesel oil biodegradation activities in fertilized soils. Biostimulation by inorganic fertilization resulted in a statistically significant ($P \leq 0.05$) increase of the total hydrocarbon loss in all five noninoculated (+F −I) soils. This could be observed already after 10 days of incubation at 10°C with most samples. With soils 1, 2, 4, and 5, the hydrocarbon loss was higher in the fertilized noninoculated (+F −I) soils than in the unfertilized inoculated (−F +I) soils (Table 4). The increase of the soil indigenous biodegradation activity after fertilization was statistically significantly ($P \leq 0.05$) higher in the carbonate-rich soils 2 and 5 than in the carbonate-free soils 1, 3, and 4 (Table 5). Biodegradation activity of the indigenous soil microorganisms (=total hydrocarbon loss minus abiotic loss) in the fertilized noninoculated soils was highest in soil 2, in which 32% of the added diesel oil was biodegraded after 20 days at 10°C; in the other four soils, the biotic decontamination due to indigenous activities was in the range of 11 to 23% (Tables 4 and 5).

In none of the five soils did fertilization plus inoculation (+F +I) result in a statistically significantly ($P \leq 0.05$) higher decontamination than fertilization alone (Table 4).

Total decontamination. The total diesel oil decontamination increased with incubation time, independent of treatment. After 20 days at 10°C, the total hydrocarbon loss was significantly ($P \leq 0.05$) higher in the fertilized soils (27 to 53%) than in the unfertilized soils (16 to 31%); the residual contamination was 3.4 to 2.3 and 4 to 3.4 g of hydrocarbons · kg (dry weight)$^{-1}$ in the fertilized and unfertilized soils, respectively. The highest decontamination of 53% (2.65 g of hydrocarbons · kg [dry weight]$^{-1}$) was obtained in fertilized soil 2 and was independent of inoculation (Table 4). There was no statistically significant differences among total hydrocarbon losses in soils 1, 3, 4, and 5 after 20 days following fertilization plus inoculation; total hydrocarbon loss was significantly ($P \leq 0.05$) higher in soil 2 than in the other four soils (Table 5).

DISCUSSION

In artificially contaminated soils, abiotic processes, such as volatilization, transformation, and adsorption to soil colloids (clay minerals and humus particles), play an important role in the apparent decontamination of mineral oil hydrocarbons. A certain part of the mineral oil hydrocarbons added to soils remains undetectable (13). In the present study, a considerable loss of hydrocarbons (ca. 20% of the contamination, independent of soil type) could be attributed to abiotic processes. Measuring the abiotic losses in artificially contaminated soils should always be carried out in conjunction with biodegradation studies (22) to avoid overestimation of the biodegradation

activity and underestimation of other possible disappearance mechanisms.

A major limitation in bioremediation of hydrocarbon-contaminated soil is the availability of nutrients. Especially with subsoils, nutrients have to be added in the form of inorganic fertilizers. Sims et al. (22) recommended a C/N/P ratio of 120:10:1. Optimal C/N ratios between 9:1 and 200:1 have been reported for waste oils and sludges (reviewed in reference 16). A large number of both laboratory and field experiments demonstrated enhancement and acceleration of oil biodegradation in fertilized soils (2, 12, 16, 18, 19). The results of the present study clearly indicate that biostimulation by inorganic fertilization significantly enhanced diesel oil biodegradation activity of the indigenous subsoil microorganisms at 10°C in all five soils investigated. We also demonstrated that biostimulation enhanced diesel oil biodegradation to a greater degree than bioaugmentation with the psychrotrophic inoculum.

The existence of a considerable subsurface microbial community that is able to metabolize a number of organic pollutants is well known. Both laboratory and field studies have demonstrated that subsurface communities can adapt to the presence of pollutants (23). The high number of diesel oil utilizers in the investigated five uncontaminated alpine subsoils (Table 3) may partly be explained by the corresponding topsoils. Through illuviation (e.g., by rain), humus components may have migrated from the topsoil into the subsoil and could have forced microorganisms to adapt their metabolism to hydrocarbons. On the other hand, the ubiquity of hydrocarbon-degrading microorganisms is well known (2).

Various authors have reported that inoculation had no positive, or only marginal, effects on oil biodegradation rates (7, 15, 26). In our study, a psychrotrophic inoculum showed only a small contribution to diesel oil decontamination in the unfertilized soils (independent of the soil properties); inoculation of the fertilized soils was without any significant effect. Biostimulation of inoculated soils by inorganic nutrients may have caused a competition between indigenous and introduced microorganisms. Possibly, the introduced microorganisms could not compete with the indigenous soil microorganisms and may have been replaced.

Microorganisms able to degrade organic pollutants in culture may fail to function when inoculated into natural environments because they may be susceptible to toxins or predators in the environment, they may use other organic compounds in preference to the pollutant, or they may be unable to move through the soil to sites containing the contaminant (8). The successful use of microbial inocula in soils requires the microorganisms to contact the contaminant. Physical adsorption to soil particles or filtration by small pores may inhibit the transport of microorganisms (5).

All five alpine subsoils investigated showed the same pattern of decontamination at 10°C: biostimulation of the indigenous soil microorganisms by inorganic fertilization was much more efficient for decontamination than was bioaugmentation with an introduced psychrotrophic inoculum. Favorable soil properties such as a near-neutral pH (reviewed in reference 2) and carbonate content, resulting in a high soil buffer capacity, may facilitate biodegradation. The addition of microorganisms (inoculation) can accelerate the initial phase of bioremediation in some cases and may be beneficial in environments in which contaminants resist biodegradation by indigenous microorganisms or when the subsurface has been sterilized by the contamination event (24).

Environmental parameters such as temperature have an equivalent or perhaps larger role than nutrients in determining the rate of degradation at contaminated sites (11). In the present study, similar decontamination rates occurred at 10°C, comparable to those previously reported at 25 to 30°C (10, 15), reflecting the adaptation of the indigenous soil microorganisms to the low temperatures that prevail in the alpine environment and in deep soil horizons. These temperatures are significantly below the optimum of mesophilic microorganisms. Cold-adapted biodegraders could be of particular importance for bioremediation treatments of the alpine and arctic areas and for in situ bioremediation treatments in deep soil horizons, where low temperatures prevail.

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