Journal of Hazardous Materials 192 (2011) 1171–1177

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The effects of carbon sources and micronutrients in whey and fermented whey on the kinetics of phenanthrene biodegradation in diesel contaminated soil

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ARTICLE INFO

Article history:
Received 15 December 2010
Received in revised form 9 June 2011
Accepted 9 June 2011
Available online 17 June 2011

Keywords:
Bioremediation
Phenanthrene
Organic amendment
Diesel
Polycyclic aromatic hydrocarbon

ABSTRACT

This paper demonstrates significant effects on phenanthrene degradation in diesel contaminated soil by the addition of organic amendments such as whey and fermented whey. Both amount of amendment added and mode of administration was shown to be decisive. There was a strong positive effect on the ^{14}C-mineralization of phenanthrene by multiple (bi-weekly) additions of fermented whey 210 mg dw kg^{-1} soil dw (FW multi) and also by single dose addition of 2100 mg dw sweet whey kg^{-1} soil dw (SW high). The most prominent effects on phenanthrene degradation kinetics were a five to fifteen fold increase in the linear growth term (k_2) and a 23–27% increase in bioavailability factor S_0 for SW high and FW multi respectively. Also, total mineralization at the end of the experiment increased from 46% in the control to 66 and 71% respectively and the lag time was reduced from 21 to 15 days by multiple addition of fermented whey. The most significant stimulating effects on phenanthrene degradation kinetics could be attributed to lactate and vitamins. This study demonstrates a more complex dependence of carbon sources and growth factors for an aromatic compound such as phenanthrene in comparison to hexadecane.

1. Introduction

Among the available soil remediation techniques, bioremediation is widely being accepted as a non-destructive and cost effective method to restore diesel-fuel contaminated soil [1]. Bioremediation techniques generally focus on optimizing the conditions for growth of oil degrading bacteria [2–6]. The addition of an extra carbon source that promotes growth of degrading organisms has proved to be an effective means to increase rate [7,8] and total degradation [7,9–12] of target pollutants in the soil. We have previously shown that fermented milk whey can accelerate the mineralization of *n*-alkanes in water [13] and enhance the degradation of *n*-hexadecane in soil [14]. We have further correlated these stimulating effects to the main components of fermented whey, i.e. lactic acid and amino acids [12]. These results indicate that the stimulating effect is mainly caused by an increased biomass of degrading microorganisms favored by the presence of easily accessible carbon sources. However, the addition of fermented whey to diesel-contaminated soil influences the mineralization kinetics of ^{14}C-hexadecane in a rather complex way, the net effect on total mineralization being stimulating or inhibiting depending on soil properties and on experimental conditions.

Diesel fuel is composed of about 80% saturated and alicyclic hydrocarbons from C_{10} to C_{20} (primarily paraffins including *n*, *iso*, and cycloparaffins), and 20% aromatic hydrocarbons (including alkylbenzenes, naphtalenes and polycyclic aromatic hydrocarbons, PAH) [6]. Since *n*-hexadecane represents a major constituent in diesel fuel it has been frequently used as a model substance in studies carried out to assess the biodegradation potential of soil pollutants and to design optimal treatments [7,15–17]. However, respiration studies using radiolabeled alkanes alone cannot be expected to predict accurately the removal of total petroleum hydrocarbons found in the polluted soil since structurally different compounds such as branched, cyclic and aromatic substances may have different degradation characteristics and thus be more or less resistant to biodegradation. Indeed poor correlation between mineralization of weathered total petroleum hydrocarbons (presumably having a low bioavailability) and the degradation of added ^{14}C-hexadecane has been demonstrated [18]. The poor correlation may however partly be attributed to the low precision of the TPH measurements which was explained by a combination of the high heterogeneity of contamination in field-collected soils, the small sample size per individual microcosm vial and the TPH microcosm method.

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doi:10.1016/j.jhazmat.2011.06.024

Monitoring the mineralization of a radiolabeled hydrocarbon added to the soil has the advantage of providing data that are accurate enough to make kinetic modeling meaningful as demonstrated by the low standard deviation between microcosm experiments carried out by us [14]. If such studies are carried out in a systematic way on structurally different constituents valuable information about the treatability of diesel-fuel contaminated soil may be gathered.

As a general rule the degradation potential of petroleum constituents are thought to be in the order *n*-alkanes > branched and cyclic alkanes > aromatics [19]. In this work we use the three ringed phenanthrene as a PAH model substance due to its individual abundance in diesel fuel [6,20]. Phenanthrene was chosen because of its high abundance in diesel fuel. The C_{14} aromatics (phenanthrene and anthracene are the two most abundant PAH:s in diesel fuel). The proposed catabolic pathway for oxidative biodegradation of phenanthrene is an initial dioxygenase-catalyzed oxidation of the arene yielding a vicinal cis-dihydrodiol. The dihydroxylated compound is then further catabolized in a series of steps leading to ring cleavage and eventually low molecular metabolites such as salicylic acid or protocatechuic acid, depending on the metabolic route [21]. Bacterial strains having the ability to aerobically degrade phenanthrene include *Pseudomonas* sp., *Rhodococcus* sp., *Flavobacterium* sp., *Beijerinckia* sp., *Mycobacterium* sp., *Mycobacterium flavescens* and *Burkholderia cepacia* [22].

The overall purpose of this study was to improve and expand the possibility of using readily available organic supplements to enhance the biodegradation of petroleum hydrocarbons in soil. Systematic studies on the stimulating effect of liquid organic amendments such as whey and fermented whey, on the degradation kinetics of a polycyclic aromatic hydrocarbon in soil have, to our knowledge, not been carried out before. The experiments were designed to study the stimulatory effect at different dosage of the two amendments and of different modes of administration; single and multidose. In addition, a designed experiment was set up where the concentration levels of the three main constituents and vitamins in fermented whey were varied. Results were evaluated in accordance with a full factorial design model.

2. Materials and methods

2.1. Soil

All experiments were carried out on a soil from the Skellefteå area in the northern part of Sweden (N64°45′, E20°57′) that had a history of petroleum hydrocarbon pollution, mainly from diesel oil. The soil had been remediated through ex situ composting to a total petroleum hydrocarbon concentration below 100 mg kg^{-1}. Thus, microorganisms having the capacity to degrade petroleum hydrocarbons were expected to be present in the soil by the time the soil sample was collected. A 10 kg sample of soil was air dried and sieved through a 2 mm mesh screen and thoroughly mixed. After sieving, the soil was classified as Sand (the coarsest class) according to US soil taxonomy. The physical and chemical characteristics of the soil are presented in Table 1. Water holding capacity (WHC) was determined gravimetrically and pH measured in a slurry with distilled water. The following measurements were performed by The Environmental Research Laboratory (Umeå, Sweden); total C and total N (elemental analyzer) and available P (Sibbesen-extraction).

2.2. Microcosms: biodegradation of phenanthrene

Biodegradation of phenanthrene was estimated by measuring the mineralization of phenanthrene-9-^{14}C (American Radiolabeled Chemicals) in to $^{14}CO_2$. Soil microcosms were prepared in 250 mL glass jars with aluminum foil lined airtight lids. Each microcosm was equipped with a 30 mL glass jar containing 10 g, dw soil and a 7 mL glass tube carbon dioxide trap containing 1 mL of 2.5 M NaOH. Soil samples were prepared as follows: 10 g dw of air dried soil was added to each jar, then 57.5 μL (50 mg) of diesel fuel spiked with 0.5 μCi phenanthrene-9-^{14}C was added and the samples were thoroughly mixed. A mineral nutrient media stock solution was prepared by dissolving 138.1 g NH_4NO_3, 31.16 g KH_2PO_4 and 31.16 g K_2HPO_4 in one liter of deionized water. Dilutions of the stock solution with and without organic amendments, were then added to give a C:N:P molar ratio of 117:11:1. Sterile control experiments with silver nitrate solution showed that abiotic mineralization was negligible. The soil humidity was adjusted to 60% of WHC which corresponded to a water content of 12% in the soil. The microcosms were kept in a closed and dark room during the 391 days of the degradation experiment. A thermostatically controlled electric radiator maintained the temperature at 25 ± 2 °C. Every 14th day the 250 mL glass jars were opened for replacement of carbon dioxide traps, adjustment of the soil humidity and for addition of organic amendments. Opening of the jars every 14th day also allowed for exchange of oxygen depleted air and was enough to prevent anaerobic conditions.

Table 1
Physical and chemical characterization of the soil sample.

	Sand
Coarse sand (250 μm–2 mm) (%)	68.0
Fine sand (20–250 μm) (%)	28.8
Silt (2–20 μm) (%)	0.8
Clay (<2 μm) (%)	2.4
WHC (g g^{-1})	0.20
pH	6.2
Total-C (mg kg^{-1})	3000
Total-N (mg kg^{-1})	200
Avail-P (mg kg^{-1})	2.10

2.3. Analysis of evolved $^{14}CO_2$

The measurement of evolved $^{14}CO_2$ followed the procedure described in earlier studies [14]. The carbon dioxide traps in the microcosms were regularly sampled for determination of trapped $^{14}CO_2$. At each sampling occasions the entire NaOH solution (1 mL) was replaced with fresh NaOH solution and the withdrawn 1 mL aliquot was mixed with 4 mL of scintillation cocktail (OptiPhase 'HiSafe' 3, Perkin Elmer) and analyzed earliest the day after on a Perkin Elmer Wallac 1414 liquid scintillation counter. The degree of mineralization was calculated as percentage of added ^{14}C-labeled carbon. The total amount of added ^{14}C-labeled hydrocarbon was determined by dissolving 11.5 μL of diesel fuel with ^{14}C-labeled hydrocarbon in 1 mL of acetone. The solution was then mixed with 4 mL scintillation cocktail and analyzed as described above.

2.4. Organic amendments

Fermented milk whey: Fermented whey (Biogen Active™, Invekta Green AB) used throughout the study was a fermentation product of sweet milk whey with a pH of 3.2. Chemical characterization was carried out in order to establish the composition of carbon sources and vitamins. These data were then used to set appropriate levels of the independent variables in the full factorial design experiment (Section 2.6). Data on the composition of fermented whey is presented in Table 2. Amino acids were analyzed

Table 2
Chemical characterization of the fermented whey used in this study.

	Concentration ($g\,kg^{-1}$)		Concentration ($\mu g\,100\,g^{-1}$)
L-Methionine	0.1	Thiamine (B1)	28.1
L-Histidine	0.1	Riboflavin (B2)	100
L-Cysteine	0.2	Pyridoxine (B6)	14
L-Glycine	0.2	Biotin (B7)	0.7
L-Tyrosine	0.2	Cyanocobalamin (B12)	0.16
L-Phenylalanine	0.2	Folic acid	4
L-Arginine	0.2	Sum of vitamins	147.0
L-Serine	0.3		
L-Threonine	0.4		
L-Proline	0.4		($g\,100\,g^{-1}$)
L-Alanine	0.4	L-lactic acid	0.935
L-Valine	0.4	D-lactic acid	0.99
L-Isoleucine	0.4	Sum of lactic acid	1.93
L-Leucine	0.6		
L-Lysine	0.6		($g\,100\,g^{-1}$)
L-Aspartic acid	0.7	Lactose	0.15
L-Glutamic acid	1.2	Dry weight (dw)	2.9
L-Ornitin	0.1		
L-Hydroxyproline	0.1		
Sum of amino acids	6.8		

Table 3
Design of experiment.

Treatment	*X*-variables (A) Lactose	(B) Lactate	(C) Vitamins	(D) Amino acids
1	−1	−1	−1	−1
2	+1	−1	−1	−1
3	−1	+1	−1	−1
4	+1	+1	−1	−1
5	−1	−1	+1	−1
6	+1	−1	+1	−1
7	−1	+1	+1	−1
8	+1	+1	+1	−1
9	−1	−1	−1	+1
10	+1	−1	−1	+1
11	−1	+1	−1	+1
12	+1	+1	−1	+1
13	−1	−1	+1	+1
14	+1	−1	+1	+1
15	−1	+1	+1	+1
16	+1	+1	+1	+1
17	0	0	0	0

The coded values for +1 corresponds to 232, 82, 18 and 0.018 mg kg^{-1} soil dw of lactate, amino acids, lactose and vitamins, respectively, −1 corresponds to no addition and 0, the center point, corresponds to 116, 41, 9 and 0.009 mg kg^{-1} soil dw of lactate, amino acids, lactose and vitamins, respectively.

by AnalyCen (Stockholm, Sweden) according to EU Dir 98/64/EC [23]. The analysis was done after hydrolysis of proteins and peptides. Concentrations in Table 2 thus correspond to the sum of respective amino acid in proteins, peptides and as free amino acid. Analysis of lactic acid, lactose, dry weight and vitamins were performed by Steins Laboratory (Loddekoping, Sweden) according to standard commercial foodstuff analysis; D-/L-lactic acid and lactose were determined using enzyme kits (D-/L-lactic acid kit, Cat. No. 1112821 and Lactose kit Cat. No. 176303 available from Boehringer-Mannheim, Germany). Vitamin B1 was extracted with hydrochloric acid, oxidized with ferric cyanide and analyzed by fluorescence analysis (excitation at 370 nm, emission at 425 nm). Riboflavin, pyridoxine, biotin, cyanocobalamin and folic acid was determined as microbiological strength by the ability to increase growth of *Lactobacillus casei* (ATCC 7469), *Saccaharomyces cereviiae* (ATCC 9080), *L. plantarum* (ATCC 8014), *L. delbrueckii* (ATCC 7830) and *L. casei* (ATCC 7469) respectively.

Sweet whey: The sweet whey used throughout this study had a dry weight of 5.38 g 100 g^{-1}, lactose 4.02 g l00 g^{-1} and lactic acid 0.16 g 100 g^{-1}. Analysis of lactic acid, lactose and dry weight were performed by Steins Laboratory (Loddekoping, Sweden) according to standard commercial foodstuff analysis.

2.5. Addition experiments

The soil was amended with fermented whey (FW) or non-fermented sweet whey (SW). Two different ways of administrating the organic amendment were tested i.e. single dose addition of respectively 210 ("low") and 2100 ("high") mg dw kg^{-1} soil dw at the beginning of the experiment and multiple dosage of fermented whey, 210 mg dw kg^{-1} soil dw every 14th day. The choice of lower and upper bounds of the organic amendment was based on previous experiments on *n*-hexadecane biodegradation with additions of fermented whey ranging from 0.2 to 2200 mg dw kg^{-1}. No stimulating effects were observed with additions lower than 220 mg dw kg^{-1} while the highest amount of amendment had significant effects including diauxic growth and a prolonged lag phase [14]. The addition of the multiple dosages every 14th day was combined with soil humidity adjustment to 60% of WHC in all microcosms. All amendments were performed in triplicate, giving a total of 18 individual experiments.

2.6. Full factorial design experiment

The effects of the parameters lactic acid, sum of amino acids, lactose and sum of analyzed vitamins were tested according to a full factorial design at two levels with a center point included. The maximum dose (+1) of each component was equivalent to its concentration in 348 mg dw fermented whey kg^{-1} soil dw, and corresponded to a soil concentration of 232, 82, 18 and 0.018 mg kg^{-1} soil dw of lactate, amino acids, lactose and vitamins respectively (Table 3). Minimum dose (−1) corresponded to no addition.

The independent *X*-variables were lactose, lactate, sum of amino acids and sum of vitamins. The following parameters were used as response variables (*Y*-variables) in the test: Lag time defined as time to 1% mineralization [24] and % mineralization at each sample point.

The factorial design experiment was performed in duplicate, giving 38 individual experiments.

2.7. Carbon sources and vitamins used in the full factorial design experiment

Amino acids, vitamins, lactate and lactose were all purchased from Sigma–Aldrich Co., Sweden. The amino acids alanine, phenylalanine, proline, tyrosine, and hydroxyproline were in BioChemica grade (≥99%), arginine and glycine were in BioChemica Ultra grade (≥99.5%) all from Fluka. Aspartic acid, cysteine, glutamatic acid, isoleucine, leucine, lysine, methionine, threonine and valine were in Reagent grade (≥98%), histidine and serine were in ReagentPlus grade (≥99%), all from Sigma-Aldrich. The vitamins thiamine, riboflavin, pyridoxine, cyanocobalamin, folic acid and biotin were all from Sigma-Aldrich with purity of respectively ≥ 99, ≥ 98, ≥ 98, ≥ 99, ≥ 98 and 99%. Lactose was in Ph Eur grade from Fluka and the D- and L-lactic acids were purchased as racemic mixture with equal amounts of D- and L- isomers (Sigma–Aldrich).

Based on the chemical characterization of the fermented whey (Table 2), four stock solutions were prepared containing 116, 102, 45 and 0.044 g L^{-1} deionized water of D/L-lactic acid, total amino acids, lactose and total vitamins respectively. These stock solutions were further diluted and added in different combinations to the soils, according to the full factorial design experiment described in Section 2.6.

2.8. Kinetic modeling used in the addition experiments

A three-half-order kinetic model assuming linear biomass growth rate for the accumulated $^{14}CO_2$-production, proposed by Brunner and Focht [25] was applied to the phenanthrene-9-^{14}C mineralization data (Eq. (1)).

$$P(t) = S_0(1 - \exp(-k_1 t - k_2 t^2/2)) + k_0 t \quad (1)$$

$P(t)$ is the cumulative $^{14}CO_2$-production (%) where t is the time (days), S_0 is the amount of substrate (%) that is highly bioavailable, k_1 is the first-order rate constant (day^{-1}), k_2 is a linear growth term (day^{-2}), and k_0 is the zero order rate constant describing the mineralization of less bioavailable substrate and indigenous mineralization (% day^{-1}).

In this experiment, the lag phase was defined as the initial time until 1% of the added ^{14}C was detected as $^{14}CO_2$ [24]. From the end of the lag phase, iterative non-linear least squares regression was performed to fit data to the model by using the SOLVER function in Microsoft Excel 2000 [26].

2.9. Statistical analysis

Addition experiments: Data were subjected to one-sided unequal variance Students t-test ($p = 0.05$) in order to test the significance of differences between treatments. Standard errors and confidence intervals of estimated parameters from the nonlinear regression analysis were determined by the use of a matrix of partial derivatives [27]. All analyses were performed in Microsoft Excel 2000.

Factorial design experiment: The results of the factorial design experiment were evaluated using the software Unscrambler 7.5 (CAMO ASA, Oslo, Norway). Separate models for each response variable (y) were fitted with multiple linear regression (MLR) according to (Eq. (2))

$$y = b_0 + \sum_i b_i x_i + \sum_{i<j} b_{ij} x_i x_j + \varepsilon \quad (2)$$

where b_0 is the intercept, x_i and x_j are the design variables representing one and two-factor interaction terms, b_i and b_{ij} are the corresponding regression coefficients and ε is the model error. The significances of the modeled main and interaction effects were subsequently determined by analysis of variance (ANOVA).

3. Results and discussion

3.1. Kinetic effects of added organic amendments

The effects of various single dose additions of fermented whey (FW), sweet whey (SW) as well as repeated addition every second week of fermented whey (FW multi) on the biodegradation of phenanthrene is illustrated in Fig. 1. The curves in Fig. 1 were fitted to the data by use of the deterministic three-half-order kinetic model assuming linear growth of degrading microorganisms as presented by Brunner and Focht [25]. The residual error was low

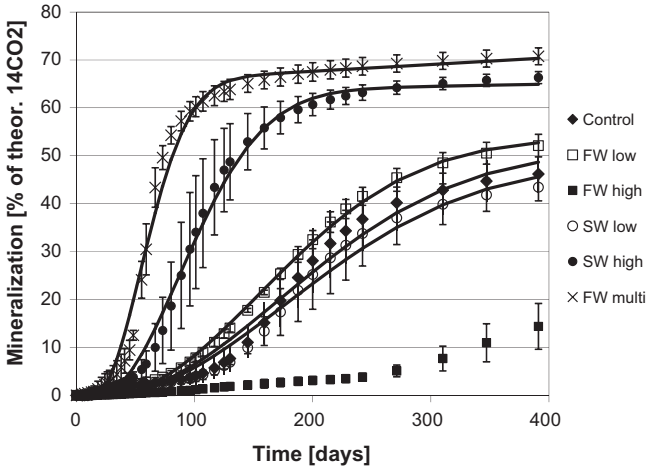

Fig. 1. Effect of added fermented whey and sweet whey on ^{14}C-phenanthrene mineralization to $^{14}CO_2$ in soil at 25 °C. FW low = Fermented whey 210 mg dw kg^{-1} soil dw; FW high = Fermented whey 2100 mg dw kg^{-1} soil dw; SW low = Sweet whey 210 mg dw kg^{-1} soil dw; SW high = Sweet whey 2100 mg dw kg^{-1} soil dw, all added at the beginning of the experiment. FW multi = Fermented whey 210 mg dw kg^{-1} soil dw added at the beginning of the experiment and then every 14th day. The curves are fitted to the data by use of the three-half-order kinetic model. Kinetic parameters are given in Table 4.

for all curves that were fit to the model (Table 4). It should be noted that the recovery of radioactive $^{14}CO_2$ proves only that there is a partial degradation of the phenanthrene molecule. Mineralization of the phenanthrene-9-^{14}C ring carbon requires however that the catabolic pathway has proceeded through several steps and, depending on the location of the initial enzymatic attack on the ring system, at least so far as to produce one or two ring (benzene or naphthalene) derivatives [21]. Thus, accumulation of such secondary metabolites during the course of the experiment cannot be precluded based on $^{14}CO_2$ data only.

The three-half-order kinetic model describes cumulative mineralization curves with a second phase of low and linear CO_2-production. Due to the very slow degradation of phenanthrene in the control and in some of the amended soils, degradation was still in the exponential phase at the end of the experiment and these curves were thus best fit to the model by setting k_0 to 0.

Apparently, the degradation pattern does not follow simple first order kinetics well. On the contrary, best curve fit was achieved by setting the first order rate constant to zero. According to Ferguson et al. [28], the first and second terms in equation 1 could represent CO_2-production from respectively a "highly bioavailable" and a "less bioavailable" fraction of the substrate. This "bimodal" degradation pattern is indicated by the two curves (FW multi and SW high) that passed the exponential phase during the 392 day experiment. In these two latter experiments the high S_0 indicates that most of the phenanthrene was rendered highly available to biodegradation by the amendments.

Table 4
Three-half-order kinetic constants for phenanthrene mineralization in soil, at 25 °C. Lag time is expressed in units of days, S_0 and mineralization in %, k_1 in day^{-1}, k_2 in day^{-2}, k_0 in % day^{-1} and R is the correlation coefficient. Values having the same superscript letters are not significantly different at a 95% level.

	Lag time (days)	S_0 (%)	k_1 (day^{-1})	k_2 (day^{-2})	k_0 (% day^{-1})	R	Mineralization after 391 days (%)
Control	21	51.3^{a}	0	4.35^{a} × 10^{-5}	0	0.973	46.3^{ab}
FW low	24	53.8^{a}	0	5.83^{b} × 10^{-5}	0	0.997	52.1^{a}
FW high	87	–	–	–	–	–	14.4^{c}
SW low	21	48.6^{a}	0	4.09^{a} × 10^{-5}	0	0.974	43.4^{b}
SW high	18	63.2^{b}	0	20.8^{c} × 10^{-5}	0.0046^{a}	0.983	66.3^{d}
FW multi	15	65.0^{b}	0	63.6^{d} × 10^{-5}	0.014^{a}	0.992	70.8^{e}

Table 5
Analysis of the effect from and within the *X*-variables lactose, lactate, amino acids and vitamins on the *Y*-variables: lag time and ^{14}C-phenanthrene mineralization (% of theoretical $^{14}CO_2$) at each sample point in soil.

Day nr															
	Lag time	5	10	15	21	26	31	38	41	45	55	67	73	80	96
A Lactose	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B Lactate	+++	NS	NS	−−	−−−	−−−	−−	NS	NS	+	++	++	++	++	++
C Vitamins	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	+	+
D Amino acid	+++	NS	−−	−−	−−−	−−−	−−−	−	−	NS	NS	NS	NS	NS	NS
AB	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AC	NS	NS	+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BD	+++	NS	−	−	−−−	−−−	−−−	−−	−s	NS	NS	NS	NS	NS	NS
CD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	107	117	125	130	145	159	173	188	200	215	243	272	311	348	392
A Lactose	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B Lactate	++	++	++	++	++	+	+	+	NS	NS	NS	NS	NS	NS	NS
C Vitamins	+	+	+	+	+	++	++	++	++	++	++	++	+	+	+
D Amino acids	NS	NS	NS	NS	−	−	−	−−	−−	−−	−−	−	−	NS	NS
AB	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BC	NS	NS	NS	NS	NS	NS	+	+	+	+	+	+	+	+	NS
BD	−	−	−	−	−	−	−−	−−	−−	−−	−−	−−	−−	−−	−
CD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS: denotes no significant effect ($p > 0.05$). The signs +, ++ and +++ denotes increasingly significant positive effect with *p*-values of 0.01–0.05, 0.001–0.01 and < 0.001, respectively. The signs −, −− and −−− denote the corresponding significant negative effects. AB, AC, AD, BC, BD and CD denote interaction effects between the *X*-variables.

There was a strong effect on the ^{14}C-mineralization of phenanthrene by multiple additions of fermented whey and to a somewhat lesser extent by single dose addition of 2100 mg dw sweet whey kg^{-1} soil dw (SW high). The effect of single dose addition of sweet whey may be somewhat surprising since not all soil microorganisms can easily utilize lactose. That specific characteristic is a common result from physiological profiling of soil microorganisms with different micro plate techniques. On the other hand, soil microorganisms as a whole community do have a high potential to utilize sweet whey and its main component lactose. Whey has been used, due to its content of easily degradable lactose, as a reductive barrier in a soil aquifer in order to deplete the available oxidants in the system [29]. It has also been shown that the number of species of soil microorganisms capable of utilizing lactose as a single substrate increases along a soil reclamation gradient [30]. That work further demonstrates that in a relatively undisturbed soil there may be a high species richness of bacteria having this capability.

The most prominent effects on phenanthrene degradation kinetics were a five to fifteen fold increase in the linear growth term (k_2) and a 23–27% increase in S_0 for SW high and FW multi respectively. Also, total mineralization at the end of the experiment increased from 46% in the control to 66 and 71% respectively. There was also a significant decrease in lag time from 21 to 15 days from multiple addition of fermented whey. The lower level addition (210 mg dw kg^{-1} soil dw) had very little effect on the degradation kinetics of phenanthrene; a slight increase of the second order rate constant was demonstrated by the addition of fermented whey (FW low) while addition of low level of sweet whey had no significant effect at all.

The addition of the high dose of fermented whey severely impeded phenanthrene degradation by increasing the lag time and apparently also inhibited the growth of degrading organisms. In an earlier experiment a similar but much smaller effect was observed on *n*-hexadecane biodegradation in sand soil by the addition of a high dose of fermented whey [14]. The effect was attributed to diauxie caused by preferential degradation of the amendment. The amendment (containing lactate 66% and lactose 5%) was totally mineralized within 10 days in both sand soil and loamy sand at 22 °C. That diauxic growth was the cause of increased lag time was supported by the fact that the length of the lag time at 22 and 7 °C respectively coincided with the degradation times of the amendment at those temperatures. In the present experiment, however, the effect cannot be explained by diauxie alone since the inhibiting effect prevails throughout the entire experiment (391 days). A tentative explanation might be that FW promotes rapid growth of microorganisms that do not degrade phenanthrene. The alternative explanation, that FW high would inhibit growth seems less likely, due to the significant stimulatory effect on *n*-hexadecane biodegradation observed by the same addition of FW in the earlier study. It seems that the observed stimulatory effect from some of the amendments is not just the promotion of quick biomass growth in general but rather of providing better conditions for organisms capable of PAH degradation. These aspects are further investigated in the section below.

3.2. Effects of carbon sources and micro nutrients

Table 5 demonstrates the different effects from the four tested X-variables in the factorial design experiment. Single variable effects were observed from lactate, vitamins and amino acids. Both lactate and amino acids were inhibitory to ^{14}C-phenanthrene mineralization at the beginning of the experiment as manifested by an increased lag time and a reduced degree of ^{14}C-phenanthrene mineralization. Amino acids were inhibitory to ^{14}C-phenanthrene mineralization also in the latter part of the experiment and showed no single variable stimulatory effect at all. The combined effect of lactate and amino acids was an increased lag time and inhibition of phenanthrene mineralization throughout a large part of the experiment.

The effect from lactate turned to stimulating at day 45 and the stimulating effect prevailed until day 188. From day 80 towards the end of the experiment vitamins showed a significant stimulatory effect on ^{14}C-phenanthrene mineralization and the combination of lactate and vitamins had a synergistic effect on ^{14}C-phenanthrene

mineralization in the latter half of the experiment, from day 173 to 348.

A similar single variable effect from lactate was observed for *n*-hexadecane mineralization in sand soil [12] i.e. an inhibition in the first part of the experiment and stimulation in the latter part. The proposed explanation for the inhibitory effect early in the experiment was that added lactate acts as a source of easily accessible carbon, which stimulates growth and leads to an increased biomass of microorganisms having a lower affinity for n-hexadecane biodegradation, and hence give rice to an inhibitory effect in the first part of the experiment. The stimulatory effect in the latter part of the experiment suggests that this increased biomass with time becomes more adapted to n-hexadecane degradation. The stimulating effect of vitamins on ^{14}C-phenanthrene mineralization observed in the present experiment indicates that the presence of even low amounts of vitamins are essential for growth of microorganisms having the enzymes capable of degrading fused ring substances such as polycyclic aromatic compounds. No such effect of vitamins was observed in the n-hexadecane experiment [12]. The biodegradation of PAH:s generally follows a more complicated metabolic pathway [3,21] compared to linear aliphatics where the main metabolic pathway is through terminal oxidation to the corresponding carboxylic acid followed by beta-oxidation to simple carboxylic acids, which are further metabolized into biomass and carbon dioxide [6,31]. Micro-growth factors such as B-vitamins may play a more critical role in the biodegradation of polycyclic aromatic substances such as phenanthrene, as indicated by the results in this study. Furthermore, the degradation of phenanthrene has been shown to produce catabolic pathway inducers, such as salicylate [21,22,32,33] that may not only increase phenanthrene degradation but also stimulate the degradation of high-molecular weight PAH:s [34]. Clearly, such metabolic feedback mechanisms should be taken into consideration in the development of better bioremediation methods for the toxic and recalcitrant five ring PAH:s.

4. Conclusion

The addition to contaminated soil of organic amendments such as fermented whey and sweet whey was shown to stimulate the mineralization of phenanthrene, both in increased linear growth rate and in the degree of total mineralization at the end of the experiment. It was also shown that the stimulating effect differed between the two organic amendments at corresponding concentrations indicating the need to find individual optimum dosage for each amendment. The importance of optimizing mode of administrating the amendments was shown by the greatly enhanced phenanthrene mineralization when fermented whey was added bi-weekly instead of only in the beginning of the experiment. The stimulating effect of complex organic amendments such as whey and fermented whey is not only an effect of easily accessible carbon but also of available micronutrients, in this case B-vitamins. The observed stimulation of phenanthrene mineralization by organic amendments is proposed to have the potential to stimulate other more recalcitrant PAHs by formation of catabolic pathway inducers.

Acknowledgments

This work was supported by the Faculty of Science, Technology and Media at Mid Sweden University.

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