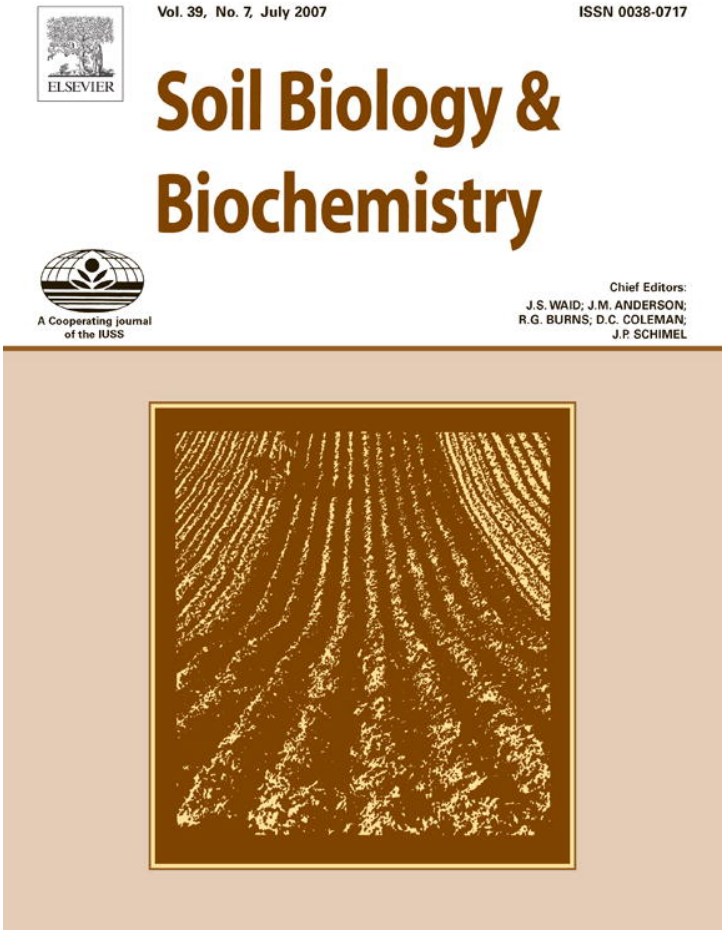

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Kinetics of microbial growth and degradation of organic substrates in subsoil as affected by an inhibitor, benzotriazole: Model based analyses of experimental results

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Abstract

Deep transport of degradable compounds through soils may occur if the metabolic activity in the soil profile is low; either by natural causes (low temperature during ice melt) or by toxic pollutants. De-icing chemicals (for roads and airports) represents a severe challenge to the soil's purifying capacity; rapid infiltration of contaminated water occurs in near-frozen soil, the contamination includes toxic compounds. Degradation experiments were conducted with toluene, and three compounds frequently used for de-icing; acetate, formate and glycol. The substrates were added to a subsoil (0.93 μmol substrate-carbon (C) g^{-1} soil, with ample amounts of nitrogen (N) and phosphorous (P)); and their mineralization was monitored in the presence of a toxic compound, benzotriazol (BTA) at various concentrations. BTA is commonly used as an additive in commercial de-icing fluids. A second and third dose of substrate was added after complete degradation of the previous one. The mineralization curves of the three consecutive doses were used to estimate kinetic parameters by fitting to a Monod-model. The model parameters estimated for each substance were the initial biomass C of the organisms growing on each substrate, C_{b0}, their maximum substrate uptake rates, V_{max}, their apparent substrate affinity, K_S, and growth yield, Y. The C_{b0} values for pristine soil were 4.9, 20.5 and 10 nmol C g^{-1} soil for formate, glycol and acetate, respectively, and 1–2 orders of magnitude lower for toluene. The K_S values were 1.1, 0.6, 2.5 and 0.13 mM for formate, glycol, acetate, and toluene, respectively. The high K_S values probably reflect diffusion limitations. The estimated yields (Y) in the absence of BTA were 0.032, 0.53 and 0.42 g biomass-C g^{-1} substrate-C for growth on formate, glycol and acetate, respectively. BTA invariably reduced the growth yield for organisms growing on the different substrates, and the yield reduction increased with increasing BTA concentrations (more than 50% reduction at 400 mg BTA l^{-1}). The degradation of the four substrates showed major differences in BTA-sensitivity, and there were strikingly weak signs (if any) of increasing BTA tolerance during growth in the presence of BTA (analyses of second and third dose experiments). The modelling of the consecutive substrate doses corroborated previous investigations of BTA effects on mineralization and community PLFA [Jia et al., 2006. Organic compounds that reach subsoil may threaten groundwater quality; effect of benzotriazole on degradation kinetics and microbial community composition. Soil Biology & Biochemistry 38, 2543–2556]. The results and the estimated Monod parameters are useful for predictive modelling of transport and degradation of pollutants as well as natural substances in sub-soils.

Keywords: Degradation; Growth; Kinetics; Model; Subsoil; Benzotriazole

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1. Introduction

Microbial degradation of soluble organic material in the vadose zone is crucial to avoid contamination of ground water aquifers, where degradable organic material may cause severe deterioration of the water quality by creating anaerobic conditions, accumulation of fermentation products and high concentrations of iron and manganese (Brown et al., 2000; Lovley and Anderson, 2000; Hansen et al., 2001; Wersin et al., 2001; Snyder et al., 2004). Organic pollutants in themselves deteriorate water quality by their bad taste/odour or toxicity (Keizer et al., 2001; Sarmah et al., 2004). The fate of organic pollutants has received much attention, and several models have been developed to analyse and predict the transport and degradation of organic compounds in the unsaturated (vadose) zone (Hunter et al., 1998; Lomander et al., 1998; French et al., 2001; Alfnes et al., 2004).

The degradation rate of organic contaminants depends on their quality as substrate for microbial growth. Repeated exposure to a contaminant which supports microbial growth will stimulate the degradation potential of the subsoil for such substances (Allen-king et al., 1994; Park et al., 2001). This implies that growth should be included explicitly in a degradation model. The need for including growth in degradation models is accentuated in all situations where the pristine soil contains few organisms which can degrade the substance in question. Surface soils contain large numbers of organisms which degrade natural substance, and for this reason, models for such systems can operate with first-order decay functions (Lundquist et al., 1999). In contrast, subsoils contain very few organisms in general, and even fewer organisms which can degrade most organic contaminants (Taylor et al., 2002). As a result, most degradation models for subsoils include growth (Alfnes et al., 2004; Mortensen and Jacobsen, 2004), even for the modelling of the degradation of naturally occurring substances (Roden and Urrutia, 1999).

Deicing fluids are potential subsoil contaminants in cold climates (Breedveld et al., 2003; Ramakrishna and Viraraghavan, 2005; Schaefer, 2006, Jia et al., 2006). Chemicals which have been used as deicers include inorganic salts and various low molecular weight organic substances. For instance, 1,2-propane diol (glycol), potassium acetate, sodium formate, and urea are widely used as runway- and aircraft de-icer/anti-icers at airports (French et al., 2001; Switzenbaum et al., 2001; Breedveld et al., 2003). As such, these compounds have a low toxicity and are readily degradable by microorganisms. Nevertheless, these compounds may penetrate deeply into the subsoil and possibly reach the aquifer, since they infiltrate rapidly during the cold season. One of the problems with commercial deicers is their additives, which may be more recalcitrant and more toxic than the main compounds (Corsi et al., 2006). One of these additives is benzotriazoles (BTA) (Cancilla et al., 1997; Breedveld et al., 2003). BTA and its derivates have been widely applied both as corrosion inhibitors in fluids and as UV light absorbers to protect polymers from photochemical deterioration (Reddy et al., 2000; Gugumus, 2002). They are applied to stabilize plastics, fibres, automotive coatings, and photographic paper (Crawford, 1999; Kuila et al., 1999), and the world production of BTA and its derivates is steadily increasing (Crawford, 1999). As a consequence, considerable amounts of benzotriazoles have leaked into the natural environment, both as direct leakage/spill from point sources and as more diffuse releases. BTA and its derivates have been found in snow, estuaries, rivers, and in groundwater (Fisher et al., 1995; Cancilla et al., 1998; Cornell et al., 2000; Reddy et al., 2000; Breedveld et al., 2003; Corsi et al., 2006). The observed toxicity of deicing chemicals has been attributed to BTA rather than the deicing agent itself (Cancilla et al., 1997; Cornell et al., 2000; Gruden et al., 2001). In addition BTA has been found to be recalcitrant to biodegradation (Rollinson and Callely, 1986; Hem et al., 2000; Tham and Kennedy, 2005; Jia et al., 2006). However, BTA is known to be light sensitive and photolytically degradable (Andreozzi et al., 1998; Hem et al., 2003). The infiltration of BTA with deicers into the subsoil may have a dual environmental effect; they represent a direct contaminant of the groundwater, and their toxicity may interfere with the degradation of other organic compounds (be it natural or man made).

On this background, we designed experiments to evaluate the effect of BTA on the degradation of a set of relevant organic pollutants found at airports. An incubation experiment with vadose soil (from 1 to 1.3 m depth) was designed, where respiration rates, microbial biomass and community composition (PLFA), were monitored as affected by substrates and different concentrations of BTA (Jia et al., 2006). In the present paper, the observed mineralization kinetics were analysed by a model of substrate uptake, growth, assimilation and respiration (mineralization). The objectives were to analyse the BTA inhibition patterns and to provide quantitative parameters for degradation rates and the inhibition of the mineralization rates of the selected compounds. Such parameters are needed in more complex transport/degradation models to predict the long-term effect on aquifers (French et al., 1999, 2001).

2. Materials and methods

2.1. Soil sampling and measurement of mineralization of first substrate dose

The soil was collected at 1–1.3 m depth from the research site Moreppen (Søvik et al., 2002) situated near Oslo International Airport, Gardermoen. A detailed description of the soil sampling and incubation strategies has been presented earlier (Jia et al., 2006). In short, four substrates; sodium formate, propylene glycol, potassium acetate (100 mg substrate-C l^{-1} soil water), and toluene (46 mg C l^{-1}) were used for the incubation experiment.

Table 1
Concentrations of added components in stock solution and final levels in the soil

Component	Mol. wt.	Conc. in stock solution ($mg\,l^{-1}$)	Final conc. in the soil moisture ($mg\,l^{-1}$)[a]	Final conc. in the soil ($\mu mole\,g^{-1}\,dw$)[a]
Mineral N and P				
Ammonium nitrate	80.65	1254	60 NH_4NO_3-N	0.23 N
Orthophosphate	136.09	1912	60 KH_2PO_4-P	0.21 P
Alternative C-substrates				
Sodium formate (F)	68.01	4111	100 formate-C	0.93 formate-C
Potassium acetate (A)	98.15	2966	100 acetate-C	0.93 acetate-C
Propylene glycol (G)[b]	76.09	1533	100 glycol-C	0.93 glycol-C
Toluene (T)	92.14	515[c]	46 toluene-C	0.43 toluene-C
Inhibitor				
1, 2, 3-Benzotriazole	119.12			
For F, A and G		0/725/1451/2901	0/100/200/400	0/0.093/0.186/0.373
For T		0/45/90/181/363/725	0/5/10/25/50/100	0/0.005/0.011/0.023/0.046/0.093

[a]The concentrations of substrates are reported as the elemental concentration of N, P and C.
[b]1,2-propane diol.
[c]The water solubility for toluene at 20 °C is $515\,mg\,l^{-1}$, therefore sufficient liquid toluene was supplied into stock solution to achieve this concentration.

For the first three substrates, five levels of BTA, 25, 50, 100, 200, and $400\,mg\,l^{-1}$ in soil moisture were supplied in soil microcosm. For toluene, lower levels of BTA 5, 10, 25, and 100 were supplied, based on preliminary experiments, which demonstrated that toluene degradation was much more sensitive to BTA than that of the other substrates. Mineral nitrogen (N) and phosphorous (P) was supplied to reach a C:N:P ratio 10:6:6, in order to avoid N- and P-limitation of microbial growth. After the supply of the solution, the soil moisture content was adjusted to 0.11 g H_2O per g dry weight, and incubation was conducted in the dark at 15 °C. Table 1 summarises the concentrations of substrates and BTA for the different treatments. The experiment was run in triplicate by incubating a full set of treatments (all BTA levels and substrates plus control soil with only BTA 0-400) three times consecutively. The three replicates were treated as blocks in the statistical analyses.

The cumulative carbon dioxide (CO_2) evolution was analysed by injecting 0.5 ml headspace samples into a He-flow through a NDIR-industrial infra-red photometer (Uras 10E, Mannesmann-Hartmann & Braun, Germany), using a gas tight syringe operated with a Gilson XX22 sampler. The volume of sample gas was replaced by He, and the dilution was taken into account when calculating accumulated CO_2 evolution. A detailed description of CO_2 sampling and analysis can be found in Jia et al. (2006). The background respiration was subtracted from the CO_2-data for the C substrate amended soils and the substrate-derived CO_2 accumulation was calculated (Jia et al., 2006).

2.2. Mineralization of second and third doses

Subsequent to the first dose (Jia et al., 2006), a second and third dose of the same substrate (but without BTA) were applied to selected samples ($100\,mg$ substrate-C l^{-1} in soil moisture for formate, glycol, and acetate, and 46 mg toluene-C l^{-1}). The timing of the second and third dose (30 and 60 d after the first) was decided based on observed CO_2 accumulation from the first dose (complete substrate depletion in all treatments; cumulative net substrate-derived CO_2 reaching a stable plateau). Prior to the new substrate addition, the samples were allowed to aerate for 2 h to restore background CO_2 levels. Thereafter, substrates and mineral nutrients were applied as a solution ($15\,\mu l\,g^{-1}$ soil dry weight). Controls received the same amount of solution containing only mineral nutrients. The background respiration measured in soil without substrates added was subtracted to estimate substrate-derived CO_2 evolution.

3. Modelling

3.1. Governing equations

A biodegradation model based on Monod kinetics was used, simulating substrate uptake, mineralization and microbial growth. The model was based on the following equations for biomass growth (C_b) and CO_2 production (C_{CO_2}):

$$\frac{dC_b}{dt} = \frac{V_{max}\,Y\,C_b\,C}{C + K_S}, \tag{1}$$

$$\frac{dC_{CO_2}}{dt} = \frac{V_{max}\,C_b(1-Y)C}{C + K_S}, \tag{2}$$

where C denotes the substrate carbon in the soil, C_b is biomass C, C_{CO_2} is substrate-derived CO_2 (cumulative), K_S is the half saturation constant, the common unit for all variables and K_S is $\mu mol\,C\,g^{-1}\,dw$; Y is the growth yield (mole C synthesized per mole of substrate-C consumed); V_{max} is the maximum substrate uptake rate ($C_{substrate}\,C_{biomass}^{-1}\,h^{-1}$).

The model was simulated in Matlab (The Math Works Inc., Mass USA). A routine was used which allowed each parameter either to have a fixed value or to be estimated by fitting model output to empirical data by minimizing the weighted squared residuals (difference between model and measurements), using the Levenberg–Marquardt method.

3.2. Model assumptions

The substrate was assumed to be readily available to the microorganisms i.e. no physical or chemical sorption or evaporation was taken into consideration. Evaporation losses were severe (and variable) for toluene, thus the yield could not be estimated for the growth on this substrate (but the model was run with a fixed default value). Mineral nutrients and electron acceptor (O_2) were considered non-limiting.

3.3. Model parameterization

Ideally, all three parameters (V_{max}, K_S, and Y) and the initial biomass (C_b), could be estimated by fitting to a single mineralization curve, but there is a degree of equifinality (i.e. different parameter combination results in similar squared residuals), and we found it necessary to use a stepwise parameterization scheme to secure stable results for meaningful comparison of treatments. In the following, all parameters estimated by model fitting will be denoted with a capped symbol (e.g. $\hat{Y}$). The scheme was somewhat different for the different substrates, depending on the type of information, which could be extracted (see below). The first step in the parameter estimation was to find the estimated growth yield based on the plateaus for cumulative substrate-derived CO_2 for the first substrate addition (see Figs. 1–3). For formate, very low (thus uncertain) growth yields were estimated, precluding any differentiation between BTA levels for this parameter. We decided therefore to use the same value for Y through all BTA levels for formate (0.032, Table 2). In contrast, the apparent growth yields on acetate and glycol were higher than for formate, and clearly reduced by increasing levels of BTA. For acetate, a CO_2 plateau is difficult to identify due to apparently substantial endogenous respiration (by the acetate-grown community). It was decided to assess $\hat{Y}$ for acetate by using the CO_2 level at the end of the incubation period, being well aware that this value depends on the length of the incubation.

In the second step the three parameters, K_S, V_{max} and C_{b0} were estimated. We found that simultaneous estimation of all three parameters for each BTA level individually would give mathematically perfect model fits, but partly unrealistic parameter values with little consistency when comparing treatments (data not shown). To overcome this problem, fixed K_S's for each substrate were used, based on the model fitting to the data for the BTA = 0 treatment. Thereafter, the V_{max} and C_{b0} values were determined for each individual BTA level.

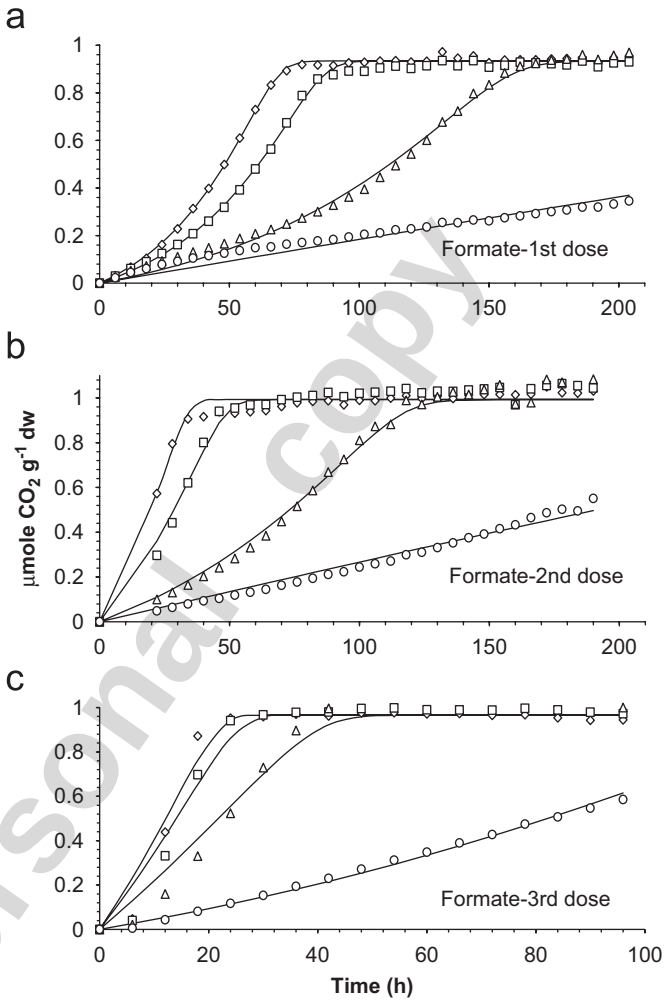

Fig. 1. Formate mineralization, measurements and simulations with a Monod model. For each BTA treatment, the result for only one of the two/three replicates is shown (for the first dose, the one with an intermediate accumulation rate; for the second and third dose, the one with the fastest accumulation rate). "◇" = BTA0; "□" = BTA100; "△" = BTA200; "○" = BTA400.

Simulation of the second and third dose of substrate was first performed by using the parameters obtained from simulating the foregoing dose, but with an initial biomass equivalent to that predicted by the modelled growth on the foregoing dose ("History" Table 3), i.e. the final biomass reached by simulating the mineralization of the first dose. Parameter estimation for the third dose followed the same routine, which can be summarized as follows:

$$C_{b0(n+1)} = C_{b0(n)} + C_0\, Y_{(n)}, \quad (3)$$

where $C_{b0(n+1)}$ is the theoretical initial biomass at the time when the $n+1$th substrate dose was added ($n = 1, 2$); $C_{b0(n)}$ is the biomass at the initiation of the degradation of the nth dose added; C_0 is the amount of substrate added each time; $Y_{(n)}$ is the estimated growth yield for growth on nth dose.

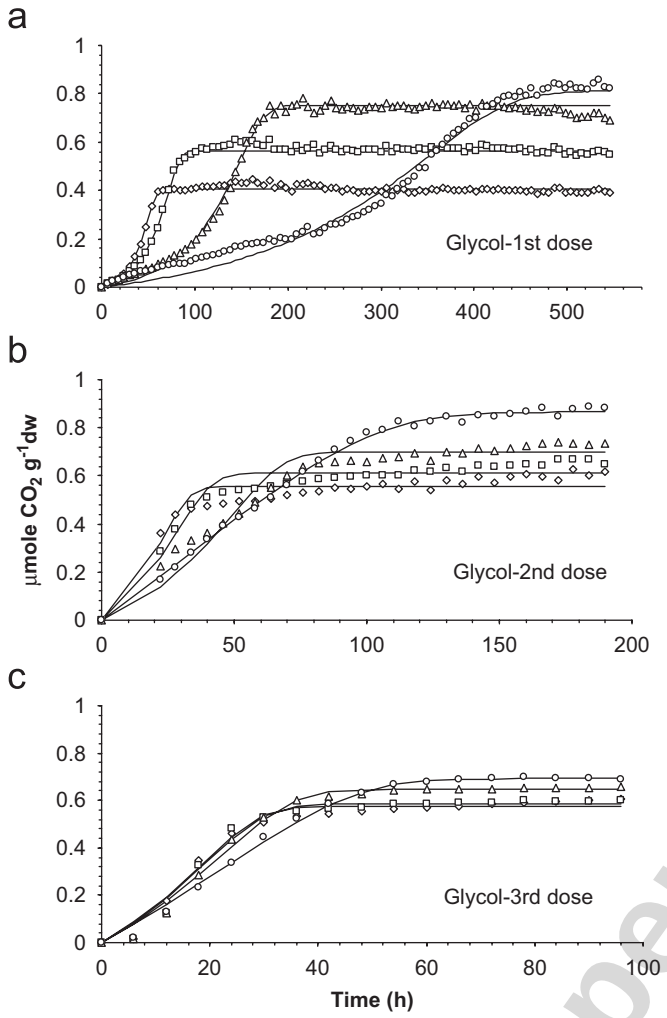

Fig. 2. Glycol mineralization, measurements and simulations with a Monod model. For each BTA treatment, the result for only one of the two/three replicates is shown (for the first dose, the one with an intermediate accumulation rate; for the second and third dose, the one with the fastest accumulation rate). "◇" = BTA0; "□" = BTA100; "△" = BTA200; "○" = BTA400.

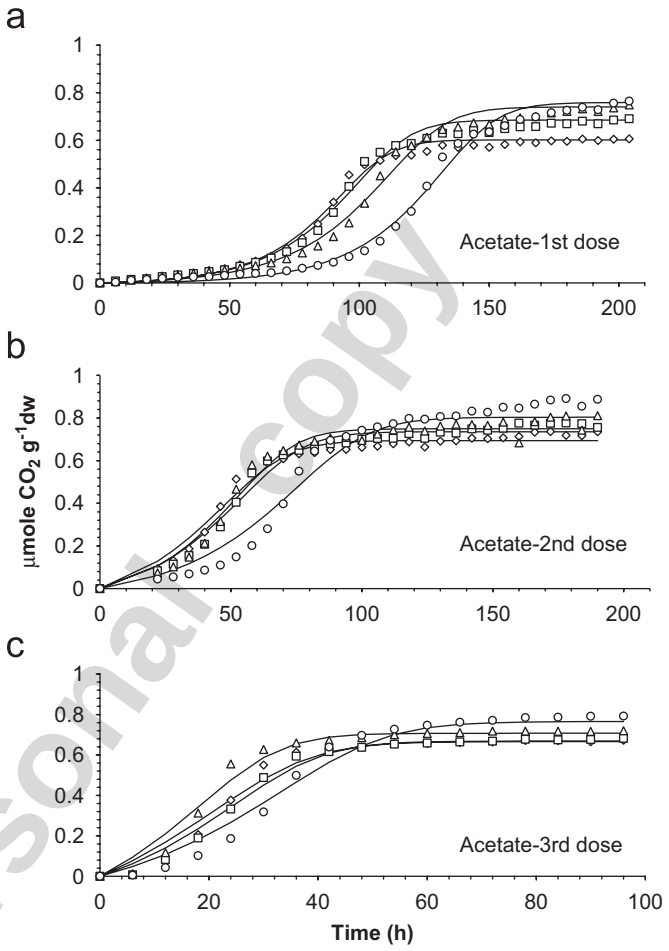

Fig. 3. Acetate mineralization, measurements and simulations with a Monod model. For each BTA treatment, the result for only one of the two/three replicates is shown (for the first dose, the one with an intermediate accumulation rate; for the second and third dose, the one with the fastest accumulation rate). "◇" = BTA0; "□" = BTA100; "△" = BTA200; "○" = BTA400.

In most cases, these initial biomass values ("History", Table 3) resulted in simulated mineralization rates, which grossly exceeded the measured rates.

We then tried to use the model-fitting procedure to estimate the initial biomass ($\hat{C}_{b0(2)}$ and $\hat{C}_{b0(3)}$), retaining the values for $\hat{Y}$, $\hat{K}_S$, $\hat{V}_{max}$ from the first substrate dose. This resulted in reasonable model fits for formate (Fig. 1 and Table 3), but poor model fits for glycol and acetate due to a clear shift in yields (results not shown). As an alternative, we allowed the model to estimate new values for $\hat{Y}$ along with initial biomass for the second and third dose of glycol and acetate, the results are shown in Table 3 and Figs. 1–3 The curves in Figs. 1a–3a show data for single flasks, one per treatment. The selected flask for presentation of each treatment was the one with intermediate mineralization rate of the three replicates (criterium: the time for 50% mineralization of the substrate should be intermediate of the three replicates). For the second and third dose, the one with the fastest mineralization for each treatment is shown ($n = 2$ for each treatment).

For toluene, the CO_2-accumulation reached stable plateaus, which were variable, due to losses of toluene by volatilization and diffusion through the rubber septa. This precluded any estimate of growth yields on toluene. It was decided to use a constant yield of 0.3 for all toluene treatments, based on observed bacterial growth yields on toluene (^{13}C-technique; Mauclaire et al., 2003), and to use individual initial substrate concentrations (i.e. toluene not lost by volatilisation) to ensure that modelled CO_2-plateaus (at simulated substrate depletion) equalled the measured plateaus for each flask. Further, we decided to use $K_S = 0.1\,\mu mol\,toluene\text{-}C\,g^{-1}$ (equivalent to $0.014\,\mu mol$ toluene g^{-1}, or $0.14\,mmol\,toluene\,l^{-1}$ soil moisture) and $V_{max} = 0.33\,mol\,C\,mol\,C_b^{-1}\,h^{-1}$, for all the toluene simulations, since this gave reasonable curve fit for most simulations (the last part of the CO_2 accumulation

Table 2
Kinetic parameters for the degradation of the first dose of formate (F), glycol (G) and acetate (A), in the presence of different concentrations of BTA (0,100, 200 and 400), obtained by model fitting to each single flask ($n = 3$ for each treatment)

Treatment	Half saturation coefficient, K_S (10^{-6} mol C g^{-1} soil)	Yield, $\hat{Y}_{(1)}$ (mol C_b mol C^{-1})	Maximum substrate uptake rate, V_{max} (mol C mol C_b^{-1} h^{-1})	Initial biomass, $\hat{C}_{b0}$ (10^{-9} mol C g^{-1} oil)	Range of correlation coefficients, r^2	Predicted half life of substrate[a] (h)
F-0	0.11	0.032±0.005	0.95±0.13	4.9±0.9	0.997±0.001	54±14
F-100	0.11	0.032[b]	0.89±0.04	3.9±1.5	0.997±0.002	66±13
F-200	0.11	0.032[b]	0.41±0.07	6.7±1.7	0.995±0.003	104±8
F-400[c]	—[c]	—[c]	—[c]	—[c]		288±48[c]
$LSD_{0.025}$			0.17	ns*	—	24
G-0	0.20	0.53±0.08	0.14±0.001	20.5±4.8	0.982±0.015	45±9
G-100	0.20	0.37±0.03	0.13±0.001	14.4±6.7	0.988±0.012	72±25
G-200	0.20	0.22±0.04	0.13±0.014	7.9±5.2	0.947±0.087	128±29
G-400	0.20	0.12±0.03	0.064±0.01	15±9.1	0.985±0.006	279±60
$LSD_{0.025}$		0.075	0.015	7		68
A-0	0.54	0.42±0.13	0.18±0.04	10±8	0.995±0.000	73±13
A-100	0.54	0.38±0.2	0.20±0.09	11±10	0.994±0.001	80±12
A-200	0.54	0.24±0.08	0.28±0.05	4±3	0.995±0.001	92±11
A-400	0.54	0.19±0.03	0.35±0.04	0.5±0.2	0.989±0.008	128±7
$LSD_{0.025}$		0.15	0.07	ns		17

Average values±SD are shown, together with $LSD_{0.025}$. *ns = not significant effect ($p > 0.05$), two-ways ANOVA.
[a]Estimated time for 50% degradation of the substrate, as predicted by the model fitted to single flask results.
[b]The value obtained for F-0 (no BTA) was used for the other BTA concentrations.
[c]Growth model could not be adapted, since the CO_2 accumulation rate declined through the whole period. The half-life of the substrate (last column) was assessed by curve inspection (i.e. the time when accumulated formate-derived CO_2–C reached 50% of the formate-C added).

Table 3
Kinetics parameters for the second and third dose experiments

	Initial biomass (10^{-9} mol C g^{-1} soil) for each period based on				X ($\hat{C}_{b0(n)}$ in % of $C_{b0(n)}$)		Yield (mol C_b mol C^{-1})		Correlation coefficient, r^2	
	(a) "History"[a]		(b) Respiration kinetics[b]							
	$C_{b0(2)}$	$C_{b0(3)}$	$\hat{C}_{b0(2)}$	$\hat{C}_{b0(3)}$	$X_{(2)}$	$X_{(3)}$	$\hat{Y}_{(2)}$	$\hat{Y}_{(3)}$	$r^2_{(2)}$	$r^2_{(3)}$
F-0	35/33	54/54.9	25/24	19/44	70/73	35/81	0.032[c]	0.032	0.980/0.977	0.997/0.976
F-100	34/32	51/48	22/19	9/47	63/58	18/97	0.032	0.032	0.996/0.987	0.997/0.985
F-200	35/38	39/41	10/127	3.2/5.6	27/31	8.1/14	0.032	0.032	0.987/0.989	0.997/0.992
F-400	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
G-0	398/385	362/491	12/139	35/182	2.9/36	9.7/37	0.40/0.40	0.38/0.38	0.988/0.871	0.745/0.983
G-100	348/319	354/453	14/111	39/182	4.1/35	11/40	0.35/0.34	0.37/0.37	0.994/0.928	0.963/0.986
G-200	189/224	357/332	77/54	64/152	41/24	18/46	0.19/0.24	0.30/0.30	0.995/0.959	0.963/0.992
G-400	142/63	259/395	26/163	132/320	18/NR	51/81	0.12/0.06	0.25/0.25	0.986/0.997	0.965/0.996
A-0	235/240	241/271	26/39	32/119	11/16	13/44	0.25/0.25	0.23/0.25	0.986/0.981	0.981/0.984
A-100	193/203	194/255.5	35/24	29/75	18/12	15/29	0.21/0.21	0.17/0.25	0.979/0.983	0.977/0.992
A-200	178/184	188/195	15/19	27/91	8.4/10	14/47	0.19/0.19	0.19/0.19	0.987/0.978	0.985/0.983
A-400	123/121	138/167	2.8/9.3	5.6/35	2.2/7.7	4.1/21	0.13/0.13	0.15/0.17	0.993/0.985	0.992/0.989

Symbols are explained in the text (lowest and highest individual numbers are shown.
NR, not recognizable.
[a]History based initial biomass was based on the simulations of the foregoing pulse (see text).
[b]Initial biomass was based on the kinetics of mineralization of the actual dose added.
[c]No attempts were made to estimate yield for individual flask, the yield value from the first dose experiment (Table 2) was used throughout.

curve before reaching a stable plateau). Thus, the only parameter estimated for comparison of toluene degradation rates (at different BTA concentrations) was the initial biomass, C_{b0}, i.e. the biomass of organisms able to degrade and grow on toluene.

All parameter estimations were performed for each single flask, and the obtained values were used as independent observations for statistical evaluation of treatment effects (two-ways ANOVA). For toluene, all single flask data are shown ($n = 2$ for each treatment).

4. Results

4.1. Experimental results

The CO_2 evolution in the control soil (with different levels of BTA) was monitored for 84 d (results not shown). Through the first 10 d, the CO_2 evolution rates were 4.9, 6.1, 6.1 and 8.0 nmol CO_2 g^{-1} soil dw for the C-0, C-100, C-200, and C-400 treatment, respectively (estimated by linear regression, $r^2 \geqslant 0.95$). The rates then declined, and more so in the BTA-amended control soils, resulting in practically identical final CO_2 accumulation values after 84 d (255 nmol CO_2 g^{-1}). The maximum observed difference in accumulated CO_2 level between C-400 and C-0 was approximately 30 nmol CO_2 g^{-1} (after 10 d of incubation) which is only 1.7% of the BTA-C added. The results thus suggest that BTA mineralization is negligible, and that the initial enhancement of CO_2-evolution by BTA is due to degradable impurities of the BTA, or enhanced endogenous respiration. The rates were much too low to affect the estimated mineralization kinetics of the (other) added substrates.

Accumulated substrate-derived CO_2-evolution for the three consecutive additions (CO_2-levels in control soils subtracted) of the three first substrates are illustrated in Figs. 1–3 together with the fitted Monod model (data for selected single flasks: the one with intermediate mineralization rate of the three replicates for the first dose, and the one with the highest mineralization rate of the two replicates for the second and third dose. In general, the substrate mineralization rates of all four substrates increased substantially through repeated additions (2. and 3. dose, note the different time scales).

The mineralization of the first dose of formate was severely inhibited by 200 and 400 mg l^{-1} BTA and this inhibition persisted through the second and third dose (Fig. 1). The estimated yield was very low (nearly all added formate-C was released as CO_2). It was decided to use the $\hat{Y}$ obtained from F-0 (0.032 mol biomass C mol^{-1} formate-C) for simulating the BTA treatments 100–400 (Table 2). The slight variations of the CO_2 plateaus were considered to be caused by a variation of the amount of added substrate. The mineralization rate of glycol (Fig. 2) was also gradually retarded by increasing concentrations of BTA, but with repeated additions the BTA effect was apparently diminished (Fig. 2b, c versus a). Another clear pattern was that the plateau of cumulative CO_2 increased with increasing BTA levels, thus it appears that a decreasing fraction of glycol-C was assimilated by the biomass (lower growth yields). As for the mineralization rates, the apparent lowering of the yield by BTA was reduced through the second and third dose.

The mineralization of acetate (Fig. 3) was less sensitive to BTA (only marginal effects except for the 400 mg l^{-1} BTA treatment); otherwise the response pattern to BTA and repeated acetate additions was similar to that for glycol regarding the apparent growth yield.

4.2. Modelling results for the first dose

The estimated parameters for the mineralization of the first dose are summarized in Table 2. The obtained $\hat{K}_S$ values (Table 2) converted to mM in the soil liquid are 1, 0.6 and 2.46 mM for formate, glycol and acetate, respectively. Table 2 also shows the predicted half-life (time taken for a 50% degradation to occur) for the four substrates. The data show a consistent BTA-effect on the degradation rate of all substrates.

The parameters for formate mineralization showed a gradual reduction in V_{max} with increasing BTA concentrations, whereas the initial biomass estimates showed no consistent BTA-response. For glycol, there was a gradual reduction in estimated growth yield, Y, with increasing concentrations of BTA, V_{max} was practically unaltered by BTA-concentrations $\leqslant 200$ mg l^{-1} (but drastically reduced at 400 mg l^{-1} BTA), and initial biomass showed no consistent response to BTA. However, attempts to keep all glycol parameters constant except for the differences in yield proved to result in poor model fits (results not shown).

For acetate, the estimated yield and initial biomass showed a gradual decline with increasing BTA levels, although the effect on initial biomass was not statistically significant. The calculated substrate half-lives (Table 2, last column) were increasing gradually with increasing BTA levels; the effects were much more severe for formate and glycol than for acetate (statistical significant effects were seen at 100–200 mg l^{-1} for formate and glycol, but only at 400 mg l^{-1} for acetate).

4.3. Modelling results for the second and third dose

The estimates of model parameters for the second and third substrate doses are shown in Table 3, and simulated respiration curves for selected flasks (fastest of two replicates for each treatment) in Figs. 1–3. The estimated yields showed some contrasts between glycol and acetate. For glycol, the yield converged for the different BTA levels through the second and third dose, i.e. the yield decreased at BTA0 and increased significantly at BTA 400. For acetate, the estimated yields were clearly lower for the second and third dose compared to that of the first dose, and this was the case at all BTA levels.

The estimated initial biomass for the second and third substrate dose were much higher than the initial values for the first dose, but not as high as one could expect based on the foregoing substrate additions and estimated yields (''History'' values, Table 3). The discrepancy is demonstrated by the expression of $\hat{C}_{b0}$ (i.e. the initial biomass estimated from the current mineralization kinetics) as percent of the final biomass based on the estimated growth on the foregoing substrate dose (X-values, column 5 and 6, Table 2). An alternative way to express the discrepancy would be to set the historical values as initial values for the subsequent mineralization period, which invariably

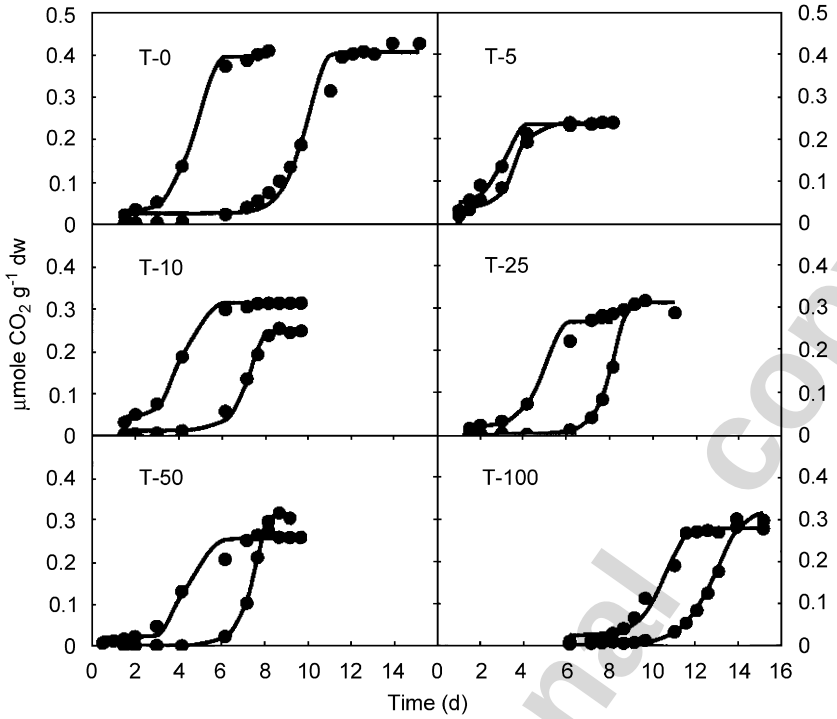

Fig. 4. Toluene mineralization, measurements and simulations with a Monod model for the first doze at BTA concentrations ranging from zero (T-0) to 100 mg l^{-1} (T-100). (both replicates at each BTA level are shown).

(except for G-400) resulted in much faster simulated mineralization rate than measured (results not shown). Regarding the initial biomass, large variation was observed between two replicates (especially for glycol and acetate) for the second and third dose, in contrast to the first dose.

4.4. Experimental and modelling results for toluene

The toluene degradation rates were highly variable for the first dose (Fig. 4), resulting in large variation between replicates with respect to the estimated initial biomass (Fig. 6). The degradation rates for the second dose was much faster than that of the first dose, showed much lower variability between replicates, and revealed a clear inhibition by BTA at a concentration of 25 mg l^{-1} (Figs. 5 and 6).

5. Discussion

The experiments illustrate a number of phenomena, which are relevant for the modelling of transport and degradation of organic contaminants in subsoils. First, the different substrates appear to be utilized by limited fractions of the entire microbial population present in the subsoil (Jia et al., 2006), as judged by the different $\hat{C}_{b0}$ in Table 2. Assuming that the dominant organisms are bacteria (which is supported by the PLFA patterns, Jia et al., 2006), we can use these values to estimate the initial numbers. Assuming that the average bacterial cell contains 3×10^{-14} g C (assuming cell volume 0.3 µm^3, Bakken and Olsen, 1987), the initial number of cells based on the C_{b0} values (Table 2) are 2, 4 and 8×10^6 cells g^{-1} soil for cells

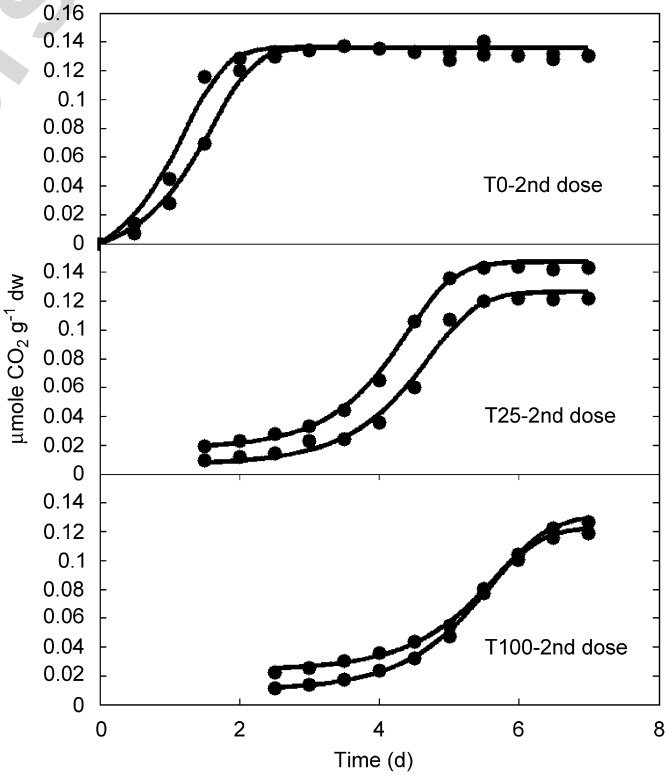

Fig. 5. Mineralization of the second dose of toluene at three BTA concentrations (0, 25 and 100 mg l^{-1}). Single flask measurements and Monod model results (both replicates at each BTA level).

growing on formate, acetate, and glycol, respectively. These numbers are 3–15 times lower than two independent estimates of total bacterial numbers in pristine subsoil:

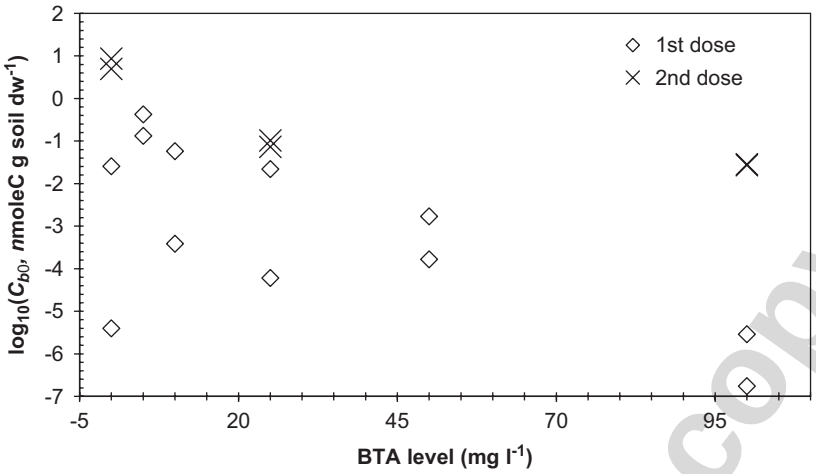

Fig. 6. Initial biomass of toluene degraders (C_{b0}) for single flasks, estimated by model fitting to the mineralization data for the first and second dose (Figs. 4 and 5).

microscopic cell counts (acridine orange staining) were $20–30 \times 10^6$ cells g^{-1} (Bakken, unpubl.), and the bacterial numbers estimated by quantification of bacterial specific PLFA were 31×10^6 cells g^{-1} (Jia et al., 2006).

The initial biomass for the toluene degraders were one to two orders of magnitude lower than that for the other substrate degraders (Fig. 6). Such relatively low numbers of toluene degraders in soil compared to that of other heterotrophs has been found by others (Allen-King et al., 1994), and reflects the composition of substrates available for microbial growth in unpolluted soils (toluene is a rather rare substrate in pristine environments compared to the other substrates).

The parameter estimates provided by the model exercise are directly relevant for anchoring parameterisation of more complex transport/degradation models if they include explicit simulation of microbial growth and substrate uptake kinetics. One example is the model used by Alfnes et al. (2004), where they simulated transport and degradation of toluene in the subsoil of the area where our samples were taken. Their V_{max} values for toluene degraders were approximately five times lower than our estimates (if corrected for the differences in temperature), but one to several orders of magnitude higher initial biomass values. Our estimated half lives of the different substrates are probably relevant first-order decay models, which are more commonly used in complex transport/degradation simulations.

The observation that final biomass-estimates from the simulation of the degradation of the first dose is much too high to be used for simulating the degradation of the second dose (Table 3) points to the necessity of limiting biomass growth in long-term modelling of frequently polluted sites. This could either be implemented by inclusion of maintenance (or death rate), or a logistic term, which sets an upper limit for the biomass, or both.

The toxicity of contaminants in the environment is often expressed as an LC_{50} value, i.e. the concentration necessary to reduce the activity (biomass or growth rate) to 50% (Bååth et al., 1998; Almås et al., 2004). The estimation of LC_{50} values are often based on short-term activity measurements, such as thymidine incorporation (Bååth, 1998; Bååth et al., 2001). However, several principally different mechanisms might be involved in the inhibitory effects of toxic compounds on microbial activity and degradation. Firstly, toxicants could reduce the substrate uptake of the active organisms (Cooney and Wuertz, 1989). Secondly, toxicants might kill a fraction of the microbial population. Thirdly, the growth yield could be affected, either by partial uncoupling of substrate mineralization from ATP generation, or by an increased maintenance energy demand (Russell and Cook, 1995). The three mechanisms would result in altered V_{max}, C_{b0}, and Y in the model as a result of BTA inhibition in the present experiment. A fourth alternative is that K_S might be altered, but it appears less than likely that a change in the K_S of the uptake enzymes can be identified in the present experiment. The estimated K_S values (mM range) are orders of magnitude higher than that of normal substrate uptake enzymes; probably reflecting that diffusion constraints in the soil dominates the substrate concentration dependency of the mineralization rates (see Harms and Bosma, 1997). This would imply that the mineralization kinetics of the substrates would not be much affected by a BTA-effect on the substrate affinity of the uptake enzymes. This is our rationale for estimating K_S only once for each substrate (i.e. for the first dose and in the absence of BTA), and to use these values for the modelling of the all other treatments.

The results (Table 2) show compellingly that BTA affected the growth yield, at least for the two substrates for which this parameter could be estimated with reasonable accuracy (acetate and glycol). For the formate degraders, the growth yield was too low to allow a direct estimation of BTA-effects on growth yield by comparing the CO_2-plateus. However, indirect evidence for a

BTA-induced reduction in growth yield can be found in the estimated initial biomass for the degradation of the second and third dose of formate; the estimates were severely reduced with increasing concentrations of BTA (Table 3). This is clearly also the case for toluene: the initial biomass for the mineralization of the second dose of toluene was severely reduced by increasing levels of BTA (Fig. 6). In summary, BTA resulted in a substantial reduction in growth yield for all the four functional groups investigated. Quantification of microbial growth by PLFA analyses demonstrated clearly that the growth yield on glycol was reduced gradually with increasing concentrations of BTA (Jia et al., 2006). The same reduction in growth yield was demonstrated for growth on the other substrates, although the results were more variable, hence not statistically significant yield depression at the lower BTA concentrations (Jia et al., 2006). In conclusion, the two approaches (PLFA and modelling of the mineralization kinetics) demonstrate that BTA results in substantial reduction of growth yield.

In contrast, the degradation rates of the first dose of formate, glycol and acetate suggest that the initial biomass was virtually unaffected by BTA $\leqslant 200\,\mathrm{mg\,l^{-1}}$. This could be taken to indicate that BTA does not exert a strong selective pressure (except for the highest BTA dose, both for formate, and glycol). However, the estimates are rather uncertain, and selective inhibition by BTA of a fraction of potentially active organisms cannot be ruled out. Similarly, the V_{max} estimates provide a relatively weak evidence for any BTA effect on this parameter (except for the highest BTA concentration). The results for the three parameters (Y, V_{max} and C_{b0}) together, however, suggests that the yield was not the only parameter which was affected by BTA, since variation of this parameter alone (in response to BTA) did not result in an adequate model fit. This conclusion is supported by the principal component analyses of the PLFA patterns from the same experiments, which demonstrated that the effect of BTA was mainly an apparently indiscriminate reduction of growth (i.e. BTA reduced growth of all PLFA's which responded positively to substrate inputs (Jia et al., 2006)), although a minor change in the second principal component suggested a selective pressure by BTA. Again, the two approaches (PLFA analyses and the present modelling exercise) show a general agreement in suggesting that the effect of BTA was a relatively indiscriminate reduction in growth yield.

The toluene degradation was much more sensitive to BTA than that of the other three substrates. The mineralization rate of the first dose was extremely variable, probably reflecting a very inhomogeneous distribution of the sparse population of toluene degrading organisms. An alternative explanation to the variability of toluene degradation (and its BTA-sensitivity as well) could be that the toluene concentrations used were high enough to exert a toxic effect on its degraders. However, this seems unlikely, since Davis and Madsen (1996) found that toluene degradation was proportional to added concentrations up to $200\,\mu\mathrm{g\,toluene\,g^{-1}}$ soil dry weight. In comparison, we applied 46 mg toluene-C $\mathrm{l^{-1}}$, which is equivalent to $5.6\,\mu\mathrm{g\,toluene\,g^{-1}}$ soil dry weight. Thus, inhomogeneous distribution of the sparse toluene degraders in the pristine soil appears to be the most plausible explanation for the variable mineralization rates of the first dose. This explanation if further strengthened by the fact that the mineralization rates of the second dose showed only marginal differences between the replicates (Fig. 5).

In summary, the results show that the organisms degrading the different substrates had very different BTA tolerance. Further, there is compelling evidence that the most important effect of BTA is to reduce the growth yield of the organisms degrading the four substrates, and only a moderate (if any) effect on the composition of the functional groups growing on the different C substrates.

A recurring experience is that microbial communities increase their tolerance to the contaminants which they are exposed to. The phenomenon is termed pollution-induced community tolerance (PICT). It is often assumed that PICT is a result of selection of tolerant species: the sensitive fraction of the community is killed, and the tolerant ones become dominant (Bååth et al., 1998). The results of the present investigation provide strikingly weak evidence for any such increase in tolerance. The exception appears to be the glycol mineralization, for which the BTA-effect on the glycol mineralization was gradually diminished through the second and third dose (Fig. 2). The modelling results suggest that this is not a true PICT phenomenon, since the gradual diminishing effect of BTA can be modelled as a gradual increase in the initial biomass (C_{b0} as estimated by respiration kinetics, Table 3) with increasing levels of BTA. This is in clear contrast to the opposite effect of BTA on growth yields, as estimated from the CO_2 plateaus (Fig. 2, and Table 3). The conflict suggests that other phenomena than the ones included in the applied growth model are important, such as cell death and predation (which again could depend on BTA).

The mechanism by which BTA reduces the growth yield is unknown, but could hypothetically be due to its chelation of divalent cations, which is particularly strong for Cu (Ramana et al., 1991). In theory, a Cu deprivation of growing cells could result in partial uncoupling, and thus lower the yield. The apparently weak selectivity of the BTA (uniform BTA effects on all the PLFA's which increased during growth on the different substrates, almost no effect on the PLFA's which did not respond to substrates), as well as the apparent lack of PICT in response to BTA, could be taken to strengthen this hypothesis, since a stronger selection of tolerant species would be expected if a more specific toxic effects were dominating.

6. Conclusions

Several mechanisms appear to be involved in the response of microbial activities to the exposure to BTA, but a recurring observation for all the four substrates

investigated is that BTA results in a substantial reduction of the growth yield. The different functional groups had very different tolerance to BTA; acetate degradation was insensitive; formate and glycol degradation showed an intermediate sensitivity, and toluene degradation was the most sensitive. Although an apparent increase in tolerance was observed for glycol degradation, the most striking observation was the persistent inhibitory effect of BTA through three consecutive doses of substrate. These findings indicate a high sensitivity of the unsaturated zone and thus groundwater aquifers for BTA contamination.

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