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Aerobic cometabolism of chloroform by butane-grown microorganisms: long-term monitoring of depletion rates and isolation of a high-performing strain

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Abstract

The focus of this microcosm study was to monitor the performances of 17 butane-utilizing microcosms during a long-term (100–250 days) aerobic cometabolic depletion of chloroform (CF). The depletion of the contaminant began after a lag-time variable between 0 and 23 days. All microcosms quickly reached a pseudo steady-state condition, in terms of biomass concentration (with an average of 9.3×10^6 CFU ml^{-1}), chloroform depletion rate (5 μmol l^{-1} d^{-1}) and butane utilization rate (730 μmol l^{-1} d^{-1}). After about 100 days of CF depletion, a sudden 5- to 7-fold increase of the chloroform rate was observed in two microcosms, where the highest amount of contaminant had been depleted. In one of these high-performing microcosms, an experiment of chloroform depletion in the absence of butane resulted in the depletion of a surprisingly high amount of contaminant (765 $\mu mol_{CF}\ kg^{-1}_{dry\ soil}$ in 2 months) and in a marked selection of a single bacterial strain. Bioaugmentation assays conducted with the biomass selected in this microcosm and with a pure culture of the selected strain immediately resulted in very high chloroform depletion rates. Preliminary results of a study conducted with resting cells of the selected strain indicated that it can degrade chloroform concentrations up to 119 μM (14.2 mg l^{-1}) without any sign of substrate toxicity, and that it is able to transform vinyl chloride and 1,1,2-trichloroethane.

Abbreviations: CF – chloroform; CFU – colony forming unit; R_c – maximum chloroform depletion rate in each pulse, $\mu mol_{CF}\ l^{-1}\ d^{-1}$; R_b – maximum butane utilization rate in each pulse, $\mu mol_{butane}\ l^{-1}\ d^{-1}$; T_y – transformation yield, defined as (CF moles degraded) : (butane moles utilized); T_c – transformation capacity, defined as (CF moles degraded) : (amount of biomass inactivated), $\mu mol_{CF}\ mg^{-1}_{protein}$ or $\mu mol_{CF}\ mg^{-1}_{dry\ cells}$; VC – vinyl chloride; 1,1,2-TCA – 1,1,2-trichloroethane

Introduction

Chlorinated aliphatic hydrocarbons (CAHs) such as chloroform (CF), trichloroethylene (TCE) and perchloroethylene (PCE) are widespread subsurface contaminants and proven or suspected carcinogens, frequently persistent in natural ecosystems and in wastewater treatment facilities (Chang & Alvarez-Cohen 1995). Several studies performed during the last 25 years demonstrated that aerobic mixed populations grown on hydrocarbons such as methane, propane, butane and

phenol can effectively degrade most CAHs by means of cometabolism (Stirling & Dalton 1979; Fogel et al. 1986; Hopkins et al. 1993; Kim et al. 1997; Arp et al. 2001). Several significant aspects of a cometabolic biodegradation process can be effectively investigated by means of a lab-scale microcosm study: the microbial acclimation time necessary for the onset of the cometabolic biodegradation process, the contaminant biodegradation rate, the extent of competition between the added primary substrate and the CAHs present, the mass of substrate required to degrade a given amount of CAHs (transformation yield) and the amount of CAHs degraded per unit of biomass inactivated as a result of the toxicity of the degradation products (transformation capacity).

Numerous studies showed that the exposition to and the degradation of chlorinated and non-chlorinated hydrocarbons induce in subsurface microbial communities significant shifts typically oriented towards the selection of a limited number of strains resistant to the contaminants present and effective in their biodegradation, with a consequent decrease in diversity. For example, Engelen et al. (1998) report that the long-term exposition of a bacterial community to a paraffin oil named Oleo determined the marked selection of one strain; Watts et al. (2001) found that the degradation of the PCB congener 2,3,4,5-chlorobiphenyl led to the selection of four strains; Shinoda et al. (2000) report that microbial acclimation was still occurring after 3 years of phenol biodegradation. A relevant consequence of these observations is that, as the bacterial population changes, also the parameters estimated from the results relative to one reactor are likely to change progressively: for example, in a 7-year study of TCE degradation by a methane-utilizing mixed culture (Smith & McCarty 1997), the authors observed remarkable increases of the transformation yield and of the transformation capacity. It is therefore important to operate lab-scale studies of CAH biodegradation for prolonged periods, so as to obtain long-term estimates of the main parameters characterizing the process and to select bacterial strains potentially utilizable in bioaugmentation treatments.

In this study, the aerobic cometabolic depletion of CF by butane-utilizing biomasses has been monitored at microcosm scale for 250 days. The use of butane as a cometabolic substrate for the aerobic degradation of chlorinated solvents is a relatively unstudied field, although the available literature points to butane as a very promising substrate for cometabolic processes (Kim et al. 1997; Hamamura et al. 1997). The observed trends relative to several key parameters are discussed, together with the results of a microbiological characterization conducted on the microcosm that gave the best depletion performances. Bioaugmentation assays conducted with mixed cultures and with a single strain isolated from the best-performing microcosm are described; the preliminary results of the characterization of the isolated strain in terms of depletion performances are also presented.

Materials and methods

Microcosms preparation and operation

Nineteen slurry microcosms were prepared using 155-ml amber serum bottles with Teflon-lined rubber septa. Each microcosm contained 25 g of a sandy soil (23.7 g of dry solids) sampled in a non-polluted aquifer, 70 ml of non-contaminated groundwater and 75 ml of headspace filled with air. To avoid bacterial contamination, bottles, caps and all tools used for preparing the microcosms were autoclaved (121 °C, 20 min). Microcosms were divided into four groups, whose initial and operational conditions are described in Table 1. Groups 1 and 2 (CF addition after a period of biomass growth on butane) can be considered representative of processes where the primary substrate-utilizing biomass develops before the introduction or the arrival of the contaminant, such as *on site* biological pump-and-treat processes or *in situ* treatments with biological active barriers, whereas group 3 (butane addition after a period of pre-exposition to CF) can be considered representative of an *in situ* treatment in which the growth substrate is introduced in the contaminated zone some time after the contamination has occurred.

As shown in Table 1, most microcosms were characterized by moderate pulse-to-pulse variations of CF initial concentration, whereas in 2 tests this parameter was progressively raised (with a maximum of 120 μM in test B23) in order to detect possible toxic effects. The desired CF concentrations were obtained by spiking a CF aqueous

Table 1. Initial concentrations of butane and CF in the 4 groups of microcosms and concise description of butane and CF subsequent additions and of bioaugmentation treatments

Group	N. of microc.	Conditions at setup			Butane and CF subsequent additions[d]; bioaugmentation treatments	Total n. of pulses depleted	
		Butane[a]	CF[b]	X[c]		Butane	CF
1	5	36	–	0.95	Following 5 pulses of only butane (36 μM), 1 spike of only CF (2.7–73 μM) was added	5	1
2	2	36	–	0.95	Following 5 pulses of only butane (36 μM), butane (36 μM) and CF (9–27 μM) were re-added in subsequent pulses, with moderate pulse-to-pulse variations of the CF initial concentration in each microcosm ($\pm 30\%$)[e]. In test B15, the butane concentration of the last 6 pulses was reduced to 14 μM	24–35	12–20
3	5	–	7–18	0.95	After 35–69 days of pre-exposition to only CF, butane (36 μM) was added. Butane (36 μM) and CF were then re-added in subsequent pulses: – tests B7, B18, B19, B22: CF initial concentrations in the pulses in the 8.4–35 μM range, with moderate pulse-to-pulse variations in each microcosm ($\pm 25\%$)[5]; in tests B7 and B19, the butane concentration of the last 6 pulses was reduced to 26 μM; – test B23: CF initial concentrations were raised from 21 to 120 μM.	8–23	4–10
4	5	36	7–18	0.95	Butane (36 μM) and CF were re-added in subsequent pulses: – tests B8, B10, B14, B16: CF initial concentrations in the pulses in the 11–30 μM range, with moderate pulse-to-pulse variations in each microcosm ($\pm 23\%$)[5]; test B16 was inoculated at day 159 with 0.2 ml of suspension (at $6.7 \cdot 10^6$ CFU ml^{-1}) from B15 (group 2); in test B16, the butane concentration of the last 10 pulses was reduced to 21 μM; – test B9: CF initial concentrations were raised from 14 to 60 μM.	13–37	4–16

[a]Initial aqueous phase concentration (μM). To convert the butane μM concentration to the total μmol of butane in the microcosm (at 21 °C), multiply by 2.62.
[b]Initial aqueous phase concentration (μM). To convert the CF μM concentration to the total μmol of CF in the microcosm (at 21 °C), multiply by 0.0825.
[c]Initial aqueous phase concentration (10^6 CFU ml^{-1}).
[d]Each spike of butane or CF was usually introduced when completion of the previous spike was detected. In some cases, a delay (with a maximum of 10 days) has occurred.
[e](Standard deviation)/(average) : average of the values calculated for each microcosm of the group.

solution (33.5 mM) into the sealed microcosms. One test of group 4 (B16) was bioaugmented at day 159, adding 0.2 ml of suspension (at a biomass concentration of 6.7×10^6 CFU ml^{-1}) sampled from test B15 (group 2) with a sterile syringe. In addition, two control microcosms were sterilized with NaN_3 57 mM and spiked with both butane (36 μM) and CF (11 μM).

All microcosms were placed on an orbital shaker (150 rpm, 20–22 °C) and were operated for a peri-

od between 100 and 250 days, except those of group 1 that were monitored only until CF delivered with the first pulse had been completely removed.

The fully aerobic condition (O_2 > 8% v/v) was periodically verified by headspace analysis, and it was maintained by additions of pure oxygen. Microcosms were periodically opened (in the absence of CF and butane) and stripped with air to eliminate dissolved carbon dioxide and possible volatile products of CF degradation.

The groundwater used in the microcosms was amended with 1.6 mM nitrate (as KNO_3) and 0.1 mM phosphate (as KH_2PO_4 and K_2HPO_4 at 0.65:1 weight ratio) at the beginning of the tests, in order to prevent nutrients from being a limiting factor in the biomass growth. Nutrients (N and P) in the microcosms were also provided every time the nitrate concentration was lower than 0.16 mM.

Analysis and chemicals

Butane (99.95%, Fluka Chemie, Buchs, CH), CF (99%, BDH Laboratory Supplies, Poole, UK), oxygen (99.5%, SIAD, Bergamo, Italy) and carbon dioxide were measured in the headspace of the microcosms. CF and butane were measured with a Hewlett Packard 6890 GC equipped with both an electron capture detector (ECD) and a flame ionization detector (FID) (injection volume 40 μl; split ratio 10:1; injector temperature 250 °C; capillary HPVOC column (30 m × 0.32 mm) operated isothermally at 135 °C; carrier gas He (2.8 ml min^{-1}); ECD and FID temperature 250 °C). Oxygen and carbon dioxide were measured with a Varian 3300 GC equipped with a thermal conductivity detector (TCD) (injection volume 500 μl; injector temperature 150 °C; packed Carbonsieve SII-SS column (3 m × 3.2 mm); oven temperature 60 °C for 5 min, 10 °C min^{-1} up to 220 °C, 220 °C for 14 min; carrier gas He (16 ml min^{-1}); TCD temperature 220 °C; filament temperature 250 °C). Nitrate concentration in the liquid phase was measured by Ion Cromatography (injection volume 20 μl) utilizing a 880-PU Jasco pump (1 ml min^{-1}, 65 kg cm^{-2}), a $NaHCO_3$ 1.7 mM + $NaCO_3$ 1.8 mM mobile phase, an IC-SepAN1 column (250 × 4.6 mm), a CDD-6A Shimadzu conductivity detector (range 1, gain 10 μS cm^{-1}, positive polarity) and an Alltech 335-SPCS suppressor. All the methods were calibrated using external standards. Butane and CF were measured in all microcosms at intervals varying between 1 and 4 days, corresponding to 2–10 analysis in each pulse, whereas oxygen and nitrate were measured only in selected microcosms and at intervals varying between 1 and 3 pulses of butane. Liquid-phase concentrations and total amounts in each microcosm were calculated on the basis of the following equilibrium partitioning coefficients: gas-phase to liquid-phase concentration ratio (dimensionless): butane 33.6 (21 °C), CF 0.135 (21 °C) or 0.206 (30 °C), O_2 30.0 (21 °C), NO_3^- 0, VC 1.26 (30 °C), 1,1,2-TCA 0.042 (30 °C); solid-phase to liquid-phase concentration ratio (l kg^{-1}, based on a 0.05% fraction of organic carbon in the solid phase; 21 °C): butane 0.245, CF 0.028, O_2 0.0014, NO_3^- 0.

Biomass measurements and microbiological characterization of the consortia

Bacterial counts were performed by taking a 100-μl sample of suspension from the microcosms, diluting it down to 10^{-6} and plating triplicate 100-μl samples of the dilutions in the 10^{-3}–10^{-6} range on R2A agar plates. Bacterial colonies grown on the plates were grouped in different clusters and were counted on the basis of their different morphologies; the viable cell concentration of each cluster was expressed as colony forming units per ml of suspension (CFU ml^{-1}). Bacterial counts were usually performed at intervals variable between 7 and 60 days, except in some of the experiments aimed at the characterization of bacteria from microcosm B15, where more frequent counts were made.

Genomic DNA extraction: 100 ml of cells were grown in R2A liquid medium, centrifuged for 10 min at 3000 g and finally re-suspended in 500 μl of TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0). 100 μl of lysozyme (2 mg ml^{-1}), 5 ml of mutanolysin (100 U μl^{-1}) and 110 μl of proteinase K (10 mg ml^{-1}) in Sodium Dodecyl Sulfate (SDS) 10% were added and the solution was incubated at 37 °C for 30 min. The solution was then purified twice with 700 ml of phenol/chloroform/isoamyl alcohol solution (25:24:1 volume ratio) and the water phase was finally precipitated with 0.7 volume of isopropanol and washed with 70% cold ethanol.

16S rDNA amplification and purification: genomic DNA (about 50 ng) was amplified with eubacterial universal primers 27F and 1525R (Lane 1991). The reaction mixture (50 μl) con-

tained template DNA, 5 μl of 10× reaction buffer, 1.5 mM $MgCl_2$, 200 μM (each) deoxy nucleotide triphosphate (dNTP), 0.1 μM (each) primer and 2.5 U of Taq DNA polymerase. DNA amplification was performed in a thermocycler (Biometra, Goettingen, Germany) with the following profile: initial denaturation for 5 min at 95 °C; 30 cycles of denaturation (1 min at 95 °C), annealing (45 s at 50 °C) and extension (1 min at 72 °C); final extension at 72 °C for 8 min. Amplified 16S rDNA was purified from each PCR reaction mixture by using a QIAquick PCR purification.

16S rDNA sequencing: partial 16S rDNA sequences (about 850 nucleotides) of strain F were obtained using 16S rDNA oligonucleotide sequencing primers 27F, 907R, 685R (Lane 1991) with a CEQ DTcS Quick start kit (Beckman Coulter Inc., Fullerton, CA, USA) following manufacturer instructions, and sequenced on a Beckman automated sequencer (Beckman Coulter Inc., Fullerton, CA, USA). Sequences were analyzed by using BLASTN (Altschul et al. 1990), FASTA (Pearson & Lipman 1988) and Ribosomal Database Project (RDP; Maidak et al. 2001) programs.

ARDRA (*Amplified Ribosomal DNA Restriction Analysis*): Two microliters of each amplification mixture were analyzed by agarose gel electrophoresis in Tris-acetate-EDTA buffer containing 0.5 μg ml^{-1} of ethidium bromide.

Characterization of bacteria from microcosm B15

In order to characterize the depletion abilities of the high-performing biomass developed in test B15 (group 2), three types of experiments were performed.

The first, performed with microcosm B15, consisted in a test of CF depletion in consecutive pulses (26–30 μM, or 2.15–2.48 total μmol in each pulse) in the absence of butane, subsequent re-introduction of butane (four pulses, 32.5 total μmol in each pulse) and final re-spike of CF (30 μM). Analogous experiments were performed – without final CF re-spike – in two "normal-performing" microcosms (B8 and B11, group 4).

The second type of experiment consisted in the inoculation of 1.7 ml of a concentrated cell suspension (2 $mg_{protein}$ ml^{-1}) of the pure strain isolated from test B15 (strain F) in two autoclaved microcosms (IB1 and IB2) amended respectively with both butane (32 μM, or 89.2 μmol) and CF (10 μM, or 0.83 μmol) and with only CF (9 μM, or 0.74 μmol). In order to prepare the concentrated pure-cell suspension, strain F was cultured at 30 °C in a mineral medium buffered with phosphate (μM: $(NH_4)_2SO_4$ 797, $MgSO_4 \cdot 7H_2O$ 244, $CaCl_2$ 132, K_2HPO_4 8902, $NaH_2PO_4 \cdot H_2O$ 5355, $FeSO_4 \cdot 7H_2O$ 22.6, $NaNO_3$ 9000, $MnCl_2 \cdot 4H_2O$ 1.52, $ZnSO_4 \cdot 7H_2O$ 0.510, H_3BO_3 1.00, $Na_2MO_4 \cdot 2H_2O$ 0.450, $NiCl_2 \cdot 2H_2O$ 0.144, $CuCl_2 \cdot 2H_2O$ 0.100, $CoCl_2 \cdot 6H_2O$ 0.100) and grown on butane as the only reduced carbon source. Cell growth was monitored by removing 1 ml of the cultures and measuring the optical density at 600 nm (OD_{600}). Cells were harvested from cultures by centrifugation (6,000 g, 10 min), washed twice with a phosphate buffer (pH 7, K_2HPO_4 8900 μM, $NaH_2PO_4 \cdot H_2O$ 5355 μM), and re-suspended at a concentration of 2 $mg_{protein}$ ml^{-1}.

The third type of experiment consisted in CF depletion assays conducted with resting cells of strain F in 11-ml serum vials sealed with Teflon-coated butyl rubber stoppers. CF (9, 52 and 119 μM, or 31, 178 and 408 nmol) was added – as a 57 mM aqueous solution – to 1 ml of phosphate buffer (pH 7, K_2HPO_4 8900 μM, $NaH_2PO_4 \cdot H_2O$ 5355 μM) and, after 30 min of shaking at 190 rpm and 30 °C, the reactions were initiated by the addition of 0.25 ml of concentrated cell suspension (0.25 mg of protein) prepared as described above but with final re-suspension at 1 $mg_{protein}$ ml^{-1} instead of 2 $mg_{protein}$ ml^{-1}. CF concentration in the headspace was measured every 40 min. Each concentration was studied in triplicate vials.

Estimation of the lag-times and maximum depletion rates

Microbial acclimation to both butane and CF was characterized in terms of lag-time for the beginning of utilization, graphically estimated from the intersection of the maximum slope line of the interpolated depletion curve with the horizontal line passing through the initial concentration value. Each pulse of butane or CF was characterized by the maximum depletion rate (R_b: μmol_{butane} l^{-1} d^{-1}; R_c: μmol_{CF} l^{-1} d^{-1}), calculated from the graph of total moles *vs.* time by dividing the maximum slope by the volume of the liquid phase. In the following, the butane and CF maximum depletion rates in each pulse are referred to as "butane rate" and "CF rate", respectively.

Results and discussion

Lag-times for the onset of the cometabolic process, and CF depletion rates in the absence of butane

Microbial utilization of butane began after a lag-time equal to 4.1 ± 1.5 days (average of all microcosms ±95% confidence interval), whereas the depletion of CF began immediately in the microcosms where a butane-utilizing biomass had already developed (groups 1 and 2), after a lag-time of 6.8 ± 2.0 days (calculated from the time when butane was added) in the microcosms characterized by a pre-exposition to only CF (group 3) and after a lag-time of 16 ± 5 days in the microcosms characterized by the contemporary introduction of butane and CF (group 4). The concentrations of butane and CF *vs.* time during the initial 60–100 days of operation are shown in Figure 1 for three representative microcosms of group 2, 3 and 4, respectively.

The concentrations of CF *vs.* time during the pulse of only CF in the five microcosms of group 1 are shown in Figure 2a. The corresponding initial CF specific depletion rates (obtained by dividing the initial rates by the biomass concentration measured in each microcosm before the addition of CF) are shown in Figure 2b together with the best-fitting simulation obtained with a Michaelis-Menten-type equation without inhibition. The non-specific depletion rate obtained at a 9.8 μM initial aqueous concentration is significantly higher (12 vs. 2.4 μmol l^{-1} d^{-1}) than the one obtained in analogous conditions (slurry microcosms with the same solid to liquid ratio, biomass growth without CF, absence of the primary substrate during the CF pulse, 8.5 μM CF initial concentration) in a previous study with a mixed propane-utilizing population (Frascari et al. 2003); this confirms that butane can be a very effective growth substrate for the cometabolic transformation of CF, as reported by other authors (Kim et al. 1997).

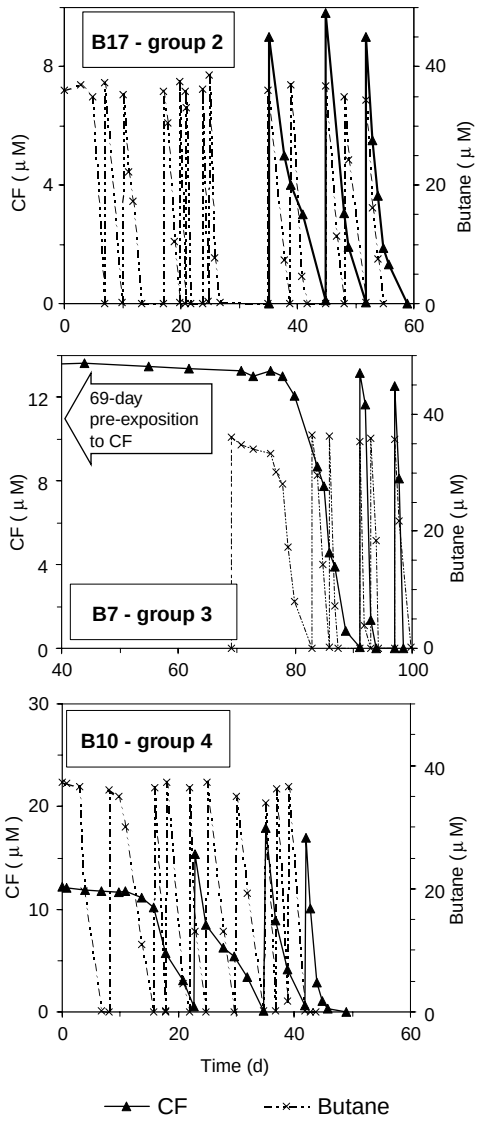

Figure 1. Concentrations of butane and CF *vs.* time during the initial 60–100 days of operation for three representative microcosms of group 2 (B17), 3 (B7) and 4 (B10) respectively. Concentration profiles in the other microcosms of each group are similar.

Long-term characterization of the cometabolic process

After 2 to 3 pulses of butane in the presence of CF, all microcosms of groups 2, 3 and 4 reached a pseudo steady-state condition, characterized by moderate pulse-to-pulse fluctuations of the biomass concentration and substrate utilization rates around a quite constant value, specific for each microcosm. CF depletion rates remained quite constant in each microcosm, with the exception of the two microcosm where the CF initial concentration of the pulses was progressively raised (as specified in Table 1). The average values of the most significant parameters characterizing the steady-state condition in these three groups of microcosms are reported in Table 2 (CF pulses

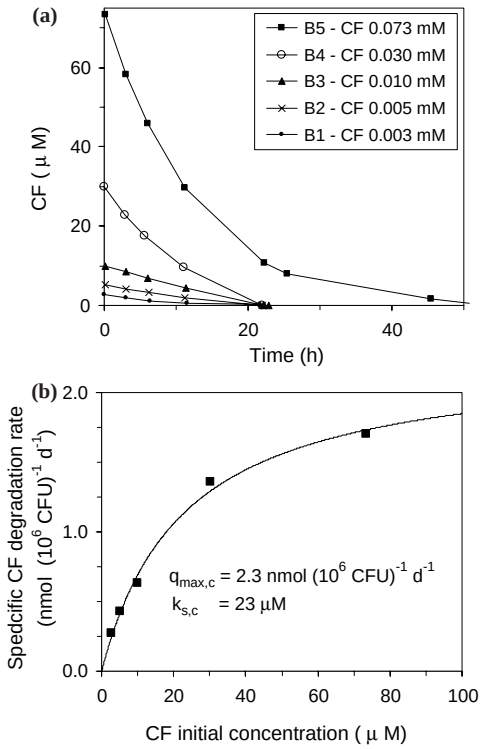

Figure 2. (a) concentrations of CF versus time during the pulse of only CF in the five microcosms of group 1; (b) corresponding specific CF depletion rates (■) with best-fitting simulation by a Michaelis-Menten-type equation (—) ($q_{max,c}$, maximum specific CF depletion rate; $k_{s,c}$, CF half-saturation constant).

depleted in the absence of butane were not included in the averages). The R_c :R_b ratio can be considered an index of the affinity of the aerobic cometabolic process for the non-growth substrate; the ratio of CF moles depleted to butane moles consumed (transformation yield, or T_y) represents an underestimation of the maximum ratio that allows to sustain the cometabolic process and can be utilized to estimate the required average substrate flow rate as a function of the contaminant flow rate entering the bioreactive zone in an *in situ* or *on site* real-scale process.

The average R_c, R_c:R_b and T_y values obtained in group 4 (biomass growth in the presence of CF) are appreciably higher than the corresponding estimates obtained in analogous conditions (slurry microcosms with the same solid to liquid ratio, biomass growth in the presence of CF, CF transformation in the presence of butane, 36 μM initial butane concentration) in a study of CF degradation by mixed butane-utilizers (Kim et al. 1997). Kim et al. (1997) found CF degradation rates varying between 0.1 and 0.3 μmol l^{-1} d^{-1} (at initial CF concentrations in the 3.5–9 μM range), 0.2–0.3% molar R_c:R_b ratios and a molar T_y equal to 0.5. In another work, Kim (1996) reports that the transformation yield and the CF degradation rates obtained in the case of biomass growth in the absence of CF are remarkably higher than the corresponding values obtained in case of growth in the presence of the contaminant, whereas this did not occur in this study.

The observed nitrogen and oxygen requirements are in good agreement with typical literature values (Rittman & McCarty 2001).

The monitoring of the CF concentration in the five microcosms of group 3 during the pre-exposition to only CF and in the sterilized controls resulted in depletion rates equal respectively to 0.7 and 0.5% of the average depletion rate measured during the pseudo-stationary phase, indicating that losses through caps, non-cometabolic biological reactions and abiotic reactions such as hydrolysis give a negligible contribution to the observed cometabolic CF depletion rates.

In test B23 (group 3), after 74 days of operation, a CF concentration of 120 μM (14.3 mg l^{-1}) was degraded without any indication of toxicity due to CF, in agreement with the findings of other authors (for example, in a study conducted with methane-utilizing resting cells, Oldenhius et al. (1990) report that CF was not toxic at 150 μM (17.9 mg l^{-1})).

Although no specific analysis were included in this study in order to detect CF degradation intermediates and to evaluate the degree of conversion of the organic chlorine to chloride ion, in a similar study of CF degradation in slurry microcosms by a mixed butane-utilizing culture (Kim et al. 1997) the cumulative ratio of Cl^- moles released to CF moles depleted increased from 2 (corresponding to a 67% conversion of organic Cl to Cl^-) to 3 (corresponding to a stoichiometric Cl^- release) during a 150-day monitoring period.

After a 100-day net time of CF depletion (time elapsed since the first exposition to the contaminant minus the sum of the periods during which no CF was present in the microcosms), two microcosms (B14 belonging to group 4, and B15 belonging to group 2) showed a sudden 4.5- to 7.4-fold increase of the CF depletion rate, with a less significant (1.8- to 2.6-fold) increase of the butane

Table 2. Average values relative to the main parameters characterizing the pseudo steady-state condition observed after 2–3 pulses of butane[a]

Operational condition	CF		Butane		CF: butane		Nitrogen	Oxygen	Biomass
	C_c^b	R_c^c	C_b^b	R_b^d	R_c:R_b (%)[e]	T_y (%)[f]	N:C (%)[g]	O:C[h]	X[i]
# 2: biomass growth in the absence of CF	14	3.9	36	610	0.70	0.56	n.a.	n.a.	9.3
# 3: biomass growth in the presence of CF, after pre-exposition to CF	17	5.9	36	960	0.99	0.59	n.a.	n.a.	13
# 4: biomass growth in the presence of CF	20	4.8	36	540	0.82	0.76	n.a.	n.a.	3.6
Overall average	18	5.0	36	730	0.85	0.67	5.1	2.43	9.3
Overall 95% conf. Interval	5	1.4	–	190	0.36	0.18	0.9	0.14	6.0

[a]The average values do not include the CF rates relative to the 2 tests where the CF concentration in the pulses was progressively raised.
[b]Initial CF or butane aqueous phase concentration in each pulse (μM).
[c]Maximum CF depletion rate in each pulse, μmol l^{-1} d^{-1} (only CF pulses in the presence of butane, 0–36 μM).
[d]Maximum butane utilization rate in each pulse (μmol l^{-1} d^{-1}).
[e]mol_{CF} mol_{butane}^{-1} (%).
[f]Transformation yield, (CF moles degraded) (butane moles utilized)$^{-1}$ (%).
[g]Nitrogen requirement, (N moles utilized) (C moles utilized)$^{-1}$ (%).
[h]Oxygen requirement (O moles utilized) (C moles utilized)$^{-1}$.
[i]Biomass concentration (10^6 CFU ml^{-1}).
n.a.: The average relative to the single groups is not available, as the N and O continuous monitoring was performed only in selected microcosms.

utilization rate. The average values estimated for the main parameters before and after the observed increase are reported in Table 3, whereas the initial CF depletion rate in each pulse for test B15 is shown in Figure 3. The increases were not accompanied by any noticeable raise in the biomass concentration, suggesting that a change in the biomass quality had occurred.

No spontaneous increase in R_c or R_b was observed in any of the other microcosms, although some were operated for longer CF depletion net times than B14 and B15 (up to 124 days). The only factor which seems to distinguish B14 and B15 from the other microcosms is the total mass of CF degraded, which was slightly higher in B14 and B15 (650 and 590 μmol $kg_{dry\ soil}^{-1}$, respectively, when the sudden R_c increase was observed) than in any of the other microcosms (where 530 μmol $kg_{dry\ soil}^{-1}$ were degraded, at the most). Other minor and fortuitous differences in the operational procedures of the 12 microcosms might have occurred.

Table 3. Performance increases observed in tests B15 and B14, and effect of the inoculation in B16 of biomass taken from test B15

	B15					B14					B16 (after inoculation)				
	C_c^a	R_c^b	C_b^a	R_b^c	T_y (%)[d]	C_c^a	R_b^c	C_b^a	R_b^c	T_y (%)[d]	C_c^a	R_b^c	C_b^a	R_b^c	T_y (%)[d]
Steady-state average value	17	5.3	36	640	0.72	20	4.0	37	500	0.73	18	3.7	36	600	0.44
Average value after the increase in performances	19	39	14	1140	2.9	28	18	34	1280	1.7	26	37	19	1830	2.7

[a]Initial CF or butane aqueous phase concentration in each pulse (μM).
[b]Maximum CF depletion rate in each pulse, μmol l^{-1} d^{-1} (only CF pulses in the presence of butane, 0–36 μM).
[c]Maximum butane utilization rate in each pulse (μmol l^{-1} d^{-1}).
[d]Transformation yield, (CF moles degraded) (butane moles utilized)$^{-1}$ (%).

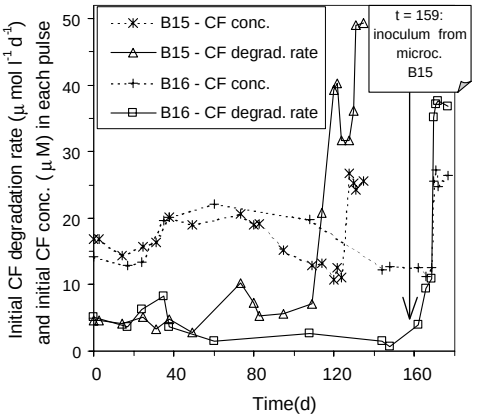

Figure 3. Microcosms B15 (group 2) and B16 (group 4): CF maximum depletion rate and CF initial aqueous concentration in each pulse (for each microcosm, time zero = time of first CF addition).

Following the inoculation of microcosm B16 (group 4) with suspension sampled from B15, both butane and CF depletion rates immediately increased and, after 10 days, a pseudo-stationary situation was achieved, with a 10-fold R_c increase, a 3-fold R_s increase and a 7-fold T_y increase with respect to the situation previous to the inoculation (Figure 3 and Table 3). As reported in a previous study (Frascari et al. 2002), these results confirm that the introduction of small amounts of selected CF-degrading biomasses in batch reactors (0.3% of the liquid volume in this experiment) can determine an immediate change in the behavior of the inoculated microcosm, which quickly reaches depletion performances similar to the parent reactor.

Characterization of bacteria from microcosm B15

The experimental data of the assay of CF depletion in the absence of butane, subsequent re-introduction of butane and final re-spike of CF, performed in B15 after the sudden improvement of the degradation performances, are shown in Figure 4, together with the data of one of the two analogous assays performed in two microcosms (B8 and B11) where no increase of the CF depletion rate had been observed. The main results of these experiments are summarized in Table 4.

Microcosm B15 has been able to maintain the CF transformation process for 60 days and for eight consecutive pulses, degrading a total CF amount 8–35 times higher than that degraded by the other two microcosms. In B8 and B11, the CF depletion process in the absence of butane determined a 17- to 34-fold decrease in biomass concentration, whereas in B15 the decrease was only 2-fold and, upon the end of the transformation process, a very high biomass concentration (3.3×10^6 CFU ml^{-1}) was measured in the microcosm aqueous phase. In all these experiments, the utilization of butane further added upon the end of the CF transformation process began after a lag-time shorter than 3 days and it was maintained for four consecutive pulses.

In B15, the depletion of CF re-spiked following the butane amendments began immediately and proceeded with a rate nearly equal to that estimated for the initial CF pulse (42 *vs.* 49 μmol l^{-1} d^{-1}), indicating a full recovery from the inactivation induced by the CF transformation in the absence of the primary substrate.

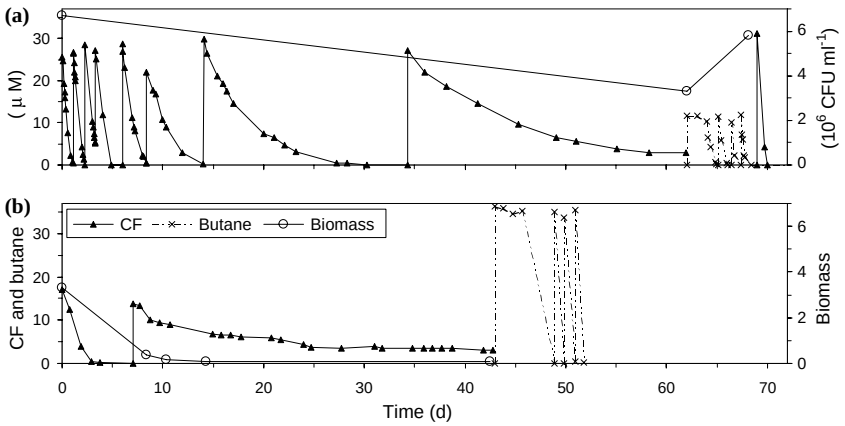

Figure 4. Microcosms B15 (a) and B8 (b): CF depletion in the absence of butane, and subsequent re-spike of butane.

Table 4. Results of the experiment of CF depletion until exhaustion in the absence of butane

Microcosm	C_c[a]	$R_{c,1st\ pulse}$[b]	$R_{c,1st\ pulse/X}$[c]	Total CF degraded[d]	Biomass decrease[e]	$T_{lag,butane}$[f]	$R_{c,respike}$[g]
B15	26	49	7.4	765	2.0	1.1	42
B8	17	8.2	2.5	98	34	2.8	n.a.
B11	7.1	0.42	0.38	22	17	0.8	n.a.

[a]Initial CF concentration (μM).
[b]Initial CF depletion rate in the 1st pulse (μmol l^{-1} d^{-1}).
[c]Specific initial CF depletion rate in the 1st pulse (nmol $(10^6$ CFU$)^{-1}$ d^{-1}).
[d]μmol_{CF} $kg^{-1}_{dry\ soil}$.
[e](Initial biomass)/(Biomass measured upon exhaustion of the depletion process).
[f]Lag-time for the onset of butane uptake after the exhaustion of the CF depletion process (d).
[g]Initial CF depletion rate in the final re-spike (μmol l^{-1} d^{-1}).
n.a.: not available, as CF was not re-spiked.

On the whole, the above data suggest that a change in the biomass quality had occurred in microcosm B15, resulting in a microbial consortium characterized by a high specific CF depletion rate and a remarkable resistance to the toxic effect of CF degradation products. The morphological characterization of the biomass samples taken from B15 indicated that the prolonged CF depletion in the absence of butane has led to a marked selection of a single bacterial strain – provisionally called F – and to the decrease of all the other culturable strains below the detection limit (30 CFU ml^{-1}). Strain F is a non-motile, Gram-positive, coccoid bacterium that forms rough, orange-pink colonies on R2A agar plates. It is able to grow on butane as a sole carbon and energy source. Partial 16S rDNA sequence analysis of the first 500 bases pairs revealed a strong homology with a recently isolated *Rhodococcus* strain able to degrade methyl t-butyl ether (Goodfellow et al. 2004).

The inoculation of strain F in the autoclaved slurry microcosm amended with both butane and CF (IB1) resulted in a drastic reduction of the lag-times relative to both compounds (with the onset of the CF depletion process within about half a day). The 1st pulse depletion rates were significantly higher than those obtained in the stationary phase of the non-inoculated microcosms (16 *vs.* 5 μmol_{CF} l^{-1} d^{-1}, and 1830 *vs.* 730 μmol_{butane} l^{-1} d^{-1}) and even higher rates (54 μmol_{CF} l^{-1} d^{-1} and 3380 μmol_{butane} l^{-1} d^{-1}) were observed after 20 pulses of both butane and CF.

The inoculation of strain F in the autoclaved slurry microcosm amended with only CF (IB2, Figure 5) resulted in the elimination of the CF

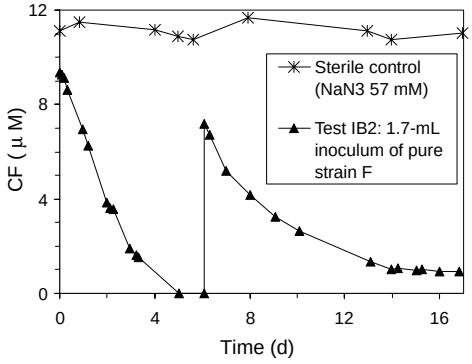

Figure 5. Depletion of CF until exhaustion in the autoclaved slurry microcosm inoculated with a pure culture of strain F.

lag-time and in the end of the transformation process after the depletion of 1.0 μmol_{CF}, corresponding to a ratio of CF transformed to biomass inactivated (T_c) of 0.29 μmol_{CF} $mg^{-1}_{protein}$. This value, that corresponds to about 0.22 μmol_{CF} $mg^{-1}_{dry\ cells}$, is in reasonable agreement with values reported by other authors for the degradation of CF by mixed-populations grown on other light aliphatic hydrocarbons (for example, Chang & Alvarez-Cohen (1995) obtained about 0.2 μmol_{CF} $mg^{-1}_{dry\ cells}$ for methane-utilizers and 0.05 μmol_{CF} $mg^{-1}_{dry\ cells}$ for propane-utilizers).

The average depletion profiles obtained in the assays conducted with resting cells of strain F are shown in Figure 6. The specific depletion rates are equal to 1.5, 2.8 and 4.7 μmol_{CF} $mg^{-1}_{protein}$ (average value in each set of triplicates), at initial CF concentrations of 9, 52 and 119 μM respectively. The rate obtained at 9 μM is in good agreement with those reported by Hamamura et al. (1997), where

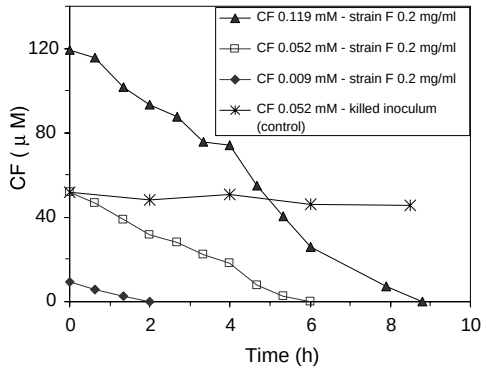

Figure 6. CF depletion by resting cells of butane-grown strain F (average depletion profiles of each set of triplicates).

rates in the range 0.7–2.5 $\mu mol_{CF}\ mg^{-1}_{protein}$ are reported for a resting-cell study of CF degradation (12 μM) by three butane-grown strains.

Further work is in progress, aimed at studying the mutual inhibition between CF and butane and the ability of the isolated strain to degrade higher CF concentrations and to transform chlorinated ethylenes and ethanes. Preliminary results indicate that the strain is able to degrade vinyl chloride (a 9.7 μM concentration, corresponding to 1.3 μmol in the vial, was completely depleted with an initial specific rate of 4.5 $\mu mol_{VC}\ mg^{-1}_{protein}$) and 1,1,2-trichloroethane (a 0.85 μM concentration, corresponding to 4.5 nmol in the vial, was completely depleted with an initial specific rate of 0.078 $\mu mol_{1,1,2\text{-}TCA}\ mg^{-1}_{protein}$).

The demonstration that the high viable biomass concentration found in microcosm B15 upon exhaustion of the CF depletion process was primarily constituted by a high-performing CF-transforming strain is in agreement with the models of cometabolism that include a term of biomass or enzyme deactivation as a result of the degradation of CAHs. Among these, the model suggested by Ely et al. (1995) includes an enzyme deactivation term expressed as a generic function of the degradation product concentration, and the model in Semprini & McCarty (1992) introduces a first-order biomass deactivation process valid only if the depletion process occurs in the absence of primary substrate utilization. However, the models developed in those two studies allow a satisfactory simulation only of the first 20 days of the test of CF depletion performed in B15, whereas for the remaining 40 days they result in depletion rates significantly higher than those measured in B15 (data not shown).

Conclusions

The long-term monitoring of the aerobic cometabolic transformation of CF by butane-utilizing biomass indicated that the process is sustainable for prolonged periods (up to 250 days). In the absence of bioaugmentation treatments, the onset of the CF depletion occurred after a lag-time variable between 0 and 23 days. CF concentrations up to 120 μM (14.3 mg l^{-1}) were degraded without any indication of toxicity due to CF. The main parameters characterizing the process (CF depletion rate, butane utilization rate, transformation yield, biomass concentration) remained basically constant during the entire monitoring period in all microcosms except 2, where a sudden 5- to 7-fold increase of the CF depletion rate and 2- to 4-fold increase of the transformation yield were observed after 100 days of CF depletion.

In one of the two high-performing microcosms the experiment of chloroform transformation in the absence of butane resulted in the depletion of a surprisingly high amount of contaminant (765 $\mu mol_{CF}\ kg^{-1}_{dry\ soil}$, corresponding to eight subsequent pulses and to a 60-day depletion period) and in a marked selection of a single bacterial strain, provisionally called F. Bioaugmentation assays conducted with the biomass selected in the above microcosm and with a pure culture of strain F immediately resulted in very high CF depletion rates and butane utilization rates. Preliminary results of a study conducted with resting cells of strain F indicated that it can degrade CF concentrations up to 119 μM (14.2 mg l^{-1}) without any sign of substrate toxicity, and that it is able to transform vinyl chloride and 1,1,2-trichloroethane.

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