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ORIGINAL PAPER

Biodegradation kinetics of the nitramine explosive CL-20 in soil and microbial cultures

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Abstract The cyclic nitramine explosive CL-20 ($C_6H_6N_{12}O_{12}$, 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) is a relatively new energetic compound which could be a persistent organic pollutant. To follow its biodegradation dynamics, CL-20 was added to soil alone or together with organic co-substrates and N-source and incubated under oxic and anoxic conditions. Without co-substrates, the CL-20 degradation was detectable only under anoxic conditions. The highest degradation rate was found under aerobic conditions and with the addition of co-substrates, succinate and pyruvate being more efficient than acetate, glucose, starch or yeast extract. When added to intact soil, CL-20 degradation was not affected by the N content, but in soil serially diluted with N-free succinate-mineral medium, the process became N-limited. About 40% of randomly selected bacterial colonies grown on succinate agar medium were able to decompose CL-20. Based on 16S rDNA gene sequence and cell morphology, they were affiliated to *Pseudomonas*, *Rhodococcus*, *Ochrobactrum*, *Mycobacterium* and *Ralstonia.* In the pure culture of *Pseudomonas* sp. MS-P grown on the succinate-mineral N(+) medium, the degradation kinetics were first order with the same apparent kinetic constant throughout growth and decline phases of the batch culture. The observed kinetics agreed with the model that supposes co-metabolic transformation of CL-20 uncoupled from cell growth, which can be carried out by several constitutive cellular enzymes with wide substrate specificity.

Keywords CL-20 · Kinetics order · Growth stoichiometry · Co-metabolism · Microbial community · Remediation strategy

The GenBank accession numbers for the 16S rRNA gene sequences obtained on this study are AY773005–AY773010. Pseudomonas sp. MS-P (=B-41417) was deposited with Agriculture Research Service Culture Collection, USA.

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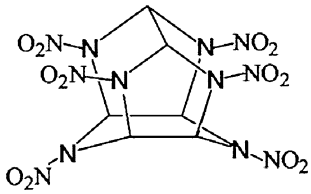
Introduction

CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) is a high-energy polycyclic



nitramine compound with a rigid caged structure.

Due to its superior explosive properties it may replace the currently used explosives such as RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) and TNT (2,4,6-trinitrotoluene). The environmental, biological, and health impacts of CL-20 and its metabolic products have recently attracted the attention of several research groups in the USA and Canada. Relative to other explosives, CL-20 appears to be non-toxic to plants and microorganisms (Gong et al. 2004; Kuperman et al. 2006; Panikov et al. 2003), but more toxic to invertebrates tested (Dodard et al. 2003; Robidoux et al. 2004).

The potential environmental impact of CL-20 has so far been evaluated by laboratory modeling studies of its migration, sorption, abiotic decomposition and biodegradation. The poor solubility of CL-20 (Monteil-Rivera et al. 2004) coupled to potentially strong binding to soil particles (Balakrishnan et al. 2004) implies a slow migration through soil and less groundwater contamination as compared with RDX and HMX. Abiotic degradation of CL-20 includes (i) thermolysis (Patil and Brill 1991), (ii) photolysis under exposure to sunlight and UV at 254–350 nm (Hawari et al. 2004), (iii) alkaline hydrolysis in vitro at pH >7.5 (Balakrishnan et al. 2003) and in alkaline soils (Crocker et al. 2005) and (iv) oxidative destruction catalyzed by clays, such as montmorillonite, biotite, illite, magnetite, and MnO_2 (Szecsody et al. 2004). Thus, CL-20 is not mobile and can be spontaneously eliminated from light-exposed or alkaline habitats, such as shallow waters or alkaline soils and sediments. However, the majority of soils and subsoils has neutral and acidic reactions and are not exposed to the sun, so CL-20 is a potentially persistent organic pollutant, and its removal depends to a great extent on microbial degraders.

Biodegradation of CL-20 in the soil was negligible without parallel addition of easily available C-compounds, e.g. succinate (Trott et al. 2003). The process was accelerated under anoxic conditions, and one of the first isolated degraders was anaerobic denitrifying bacterium *Pseudomonas* sp. FA1 (Bhushan et al. 2003). Further studies revealed various anaerobic and aerobic species including bacteria such as *Pseudomonas* (Bhushan et al. 2003, 2004a), *Clostridium* (Bhushan et al. 2004b) and *Agrobacterium* (Trott et al. 2003) as well as the fungi *Phanerochaete chrysosporium* and *Iprex lacteus* (Fournier et al. 2006). In most of these studies, the isolated degraders were said to be specialized microorganisms using CL-20 as the only N-source and their catabolic enzymes were inducible by the presence of this compound in the medium.

On the other hand, complete degradation of CL-20 to the same or similar mixture of products (NO_2^-, N_2O, NH_4^+ and formic acid) has been achieved by using pure enzymes, mainly dehydrogenases: salicylate 1-monooxygenase from *Pseudomonas* sp. (Bhushan et al. 2004a), nitroreductase from *Escherichia coli* (Bhushan et al. 2004), dehydrogenase from *Clostridium* sp. (Bhushan et al. 2005), and manganese peroxidase from white-rot fungus *Nemalotoma frowardii* (Fournier et al. 2006).

The abiotic (photolysis, mineral catalysis) and enzymatic degradation produced a similar mixture of compounds, the only difference being occasional detection of glyoxal as a specific product of enzymatic reactions. The enzymes used in the referred biodegradation studies were commercially available purified proteins isolated from microorganisms grown on standard media without CL-20.

All these facts led us to hypothesize that biodegradation of CL-20 occurs as a co-metabolic transformation catalyzed by constitutive enzymes (various dehydrogenases) with wide substrate specificity. The organisms responsible are not specialized degraders and they do not require

CL-20 as growth substrate. Therefore, first, biodegradation of CL-20 does not provide any selective advantage to degraders in situ and, second, the ability to degrade CL-20 should be widely distributed in nature.

To test these premises, we did a screening of soil microorganisms for their ability to degrade CL-20 and studied the biodegradation kinetics in soil and batch cultures of isolates with focus on the relationship between decomposition dynamics and cell growth.

Materials and methods

Chemicals

The CL-20 (purity >99%) ϵ-polymorph used in these studies was obtained from ATK Thiokol Propulsion (Brigham City, Utah) through Picatinny Arsenal, NJ, USA. Starch (soluble powder 'for iodometry', certified ACS) was obtained from Fisher; other chemicals of a purity grade of 99% and higher were obtained from Sigma.

Because of the low solubility of CL-20 in water, in most published works it was added to a growth medium dissolved in methanol or acetone or as Tween-80 stabilized suspension (Bhushan et al. 2003; Trott et al. 2003). Here we avoided use of detergents and solvents since they can serve as additional C-sources or inhibit microbial growth. Instead, a stable suspension of CL-20 was obtained by mechanical dispersion of ground CL-20 in aluminum oxide mortar with subsequent ultra sounding for 20 min and filtration (Whatman filter paper N 41). All steps were done aseptically in the laminar-flow cabinet with prior autoclaving of all hardware and membrane filtration (Millipore, 0.2 μm) of accessory solutions. Sterility of CL-20 suspension was tested by plating on nutrient agar. The actual CL-20 concentration in the resulting suspension varied between 90 and 230 μM or 40–100 mg l^{-1}.

Incubation experiments with soil

The soil samples were collected from Palisades Interstate Park, NJ under birch forest from the top layer 2–10 cm. The content of total N and organic C was respectively 0.9 and 4.1%; pH_{H_2O} was 4.9. Soil was sieved through 2 mm and stored at 4°C in sealed polyethylene bags before experiments.

In the first incubation experiment, we tested the effect of several co-substrates on the rate of CL-20 decomposition. One gram of soil in 6 ml glass vials (Canberra Co.) was mixed with 1.0 ml of liquid nutrient solution containing CL-20 (60 mg l^{-1}) and one of the following co-substrates: Na-succinate, Na-acetate, Na-pyruvate, D-glucose, starch or yeast extract. All C-sources were brought to the same C-content equivalent to 4.0 mg C g^{-1} soil and adjusted to pH 6.5–7.0 with 1 M H_3PO_4. The mineral base solutions were added containing K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$ at final concentrations of 2.0, 1.0 and 1.0 mg (g soil)$^{-1}$, respectively.

In the second experiment, the dynamics of CL-20 degradation were monitored with Na-succinate as co-substrate under oxic (air) or anoxic conditions (Argon, Matheson Gas Products, ultra high purity grade). $(NH_4)_2SO_4$ was omitted because by this time we had found that soil provided N-source strength comparable with standard nutrient media: the total soil N and NH_4^++NO_3^- contents determined by standard technique (Williams et al. 1995) were as high as 9.0 and 1.1 mg (g soil)$^{-1}$, respectively.

In both experiments, the incubation was done at 28°C in the dark. To follow degradation dynamics, 2–5 out of twenty replicate vials were sacrificed every 2–3 days. CL-20 was extracted 1 h with 2.0 ml of acidified acetonitrile (Monteil-Rivera et al. 2004), centrifuged 10 min at 16,000*g*, and analyzed by HPLC.

Sterile control: microbial activity was inactivated by soil autoclaving for 60 min at 121°C, CL-20 was added aseptically after autoclaving and the incubation and analysis of residual CL-20 were done as described above. Sterility was tested by occasional plating on nutrient agar. Two other controls were used in both incubations: soil + CL-20 in mineral base solution without co-substrate and CL-20 mixed with sterile N-free mineral base solution without soil (control for spontaneous degradation).

Incubation experiments with soil dilutions

Two grams of soil were homogenized by gentle grinding with a rubber pestle in a mortar with 2 ml of sterile water. The starting 10^{-1} dilution was prepared by mixing 0.2 ml of soil suspension (=160 ± 20 mg dry soil) with 2 ml of sterile N(–) or N(+) mineral medium of the following composition (g l^{-1}): Na-succinate, 5.0, KH_2PO_4, 6.0; K_2HPO_4, 3.0; $MgSO_4$, 0.4; $CaCl_2{\times}2H_2O$, 0.1; Na-EDTA—0.01, $FeCl_3{\times}6H_2O$—$1{\times}10^{-3}$, KI—$2{\times}10^{-4}$, $CoCl_2{\times}6H_2O$—$2{\times}10^{-4}$, $MnCl_2{\times}4H_2O$—$8{\times}10^{-4}$, $ZnSO_4$—$8{\times}10^{-4}$, H_3BO_2—$1{\times}10^{-4}$, $Na_2MoO_4{\times}2H_2O$—$1{\times}10^{-4}$, $CuCl_2$—$1{\times}10^{-4}$, $NiCl_2{\times}6H_2O$— $2{\times}10^{-4}$; CL-20—0.015–0.055. Concentration of $(NH_4)_2SO_4$ in N(+) experiments was 1.0 g l^{-1}. The following serial dilutions were prepared by transferring 0.2 ml of soil suspension from one test tube to another. Every dilution was prepared in 10 replicates. After various period of incubation two replicate test tubes were sacrificed for analysis of residual CL-20. As control, we used sterile nutrient medium + CL-20 with water instead of soil suspension.

Isolation and identification of microorganisms degrading CL-20

The standard 10-fold serial dilutions of soil were prepared and plated on complete N(+) Na-succinate-mineral medium (see above) with 2% agar added. The developing bacterial colonies were randomly isolated, grown on the liquid N(+) Na-succinate-mineral medium and checked for ability to degrade CL-20 as described above.

The isolates were examined with a Leica DMLB microscope and identified based on Gram staining, acid resistance, cultural features and 16s rDNA gene sequencing. DNA extraction, PCR amplification and sequencing was done as described before (Sizova et al. 2003). Amplified PCR products were sequenced by Applied Biosystems cycle sequencing technology; sequences were determined on an ABI PRIZM R 3100 Genetic Analyzer (GTSF, Michigan State University). The screening for similarity was carried out with BLAST (http://www.ncbi.nlm.nih.gov/blast) and the Taxonomic Hierarchy Model from Michigan State University (http://rdp.cme.msu.edu/classifier/) (Cole et al. 2003).

Cultivation of isolates

The biodegradation of CL-20 in *Pseudomonas* sp. MS-P culture was followed in two experiments. In the first one, N(+) Na-succinate liquid medium containing CL-20 suspension was distributed by 2 ml in 20 cupped vials, inoculated with 10^9 bacterial cells and incubated under continuous shaking (180 rpm) at 28°C. Optical density D_{600} and residual CL-20 were analyzed in 2–5 sacrificed vials as described above. The control vials contained no cells.

In the second experiment we tested whether CL-20 is degraded by non-growing cells present in the stationary phase of the batch culture at zero residual concentration of succinate. *Pseudomonas* sp. MS-P was grown on medium containing 10 g l^{-1} of succinate until cell density reached maximum and succinate concentration dropped below 100 mg l^{-1} (analyzed by HPLC). Then the content of the flask was aseptically split into three equal parts and mixed with CL-20 and (i) mineral base solution (no succinate), (ii) base solution + 10 g l^{-1} of Na-succinate and (iii) base solution + 20 g l^{-1} of Na-succinate. Then all three mixtures were distributed by 2 ml in series of 20 vials each and incubated as described before. The culture (i) was stationary and contained non-growing cells, while cultures (ii) and (iii) were allowed to continue to grow and served as positive controls for CL-20 biodegradation.

Analytical methods

The turbidity of cell cultures was measured at 600 nm with spectrophotometer Ultrospec-1000 (Pharmacia Biotech) and converted to cell dry weight. The conversion factor was determined by wet oxidation of cell suspension to CO_2 (TOC-VE, Shimadzu) assuming the average 46% cell carbon (Erickson et al. 1978).

The CL-20 concentrations were determined by HPLC (Larson et al. 2002; Trott et al. 2003) with a Shimadzu instrument on a C_{18} Zorbax column (Rocland Technologies, DuPont) 4.6 mm × 25 cm. Succinate was analyzed by HPLC on Zorbax

SB phenyl column 4.6 mm × 25 cm with 0.01 N H_2SO_4 as mobile phase, 0.5 ml/min, UV-detection at 200 nm.

Statistical calculations and kinetic data analysis

Descriptive statistics of primary data including mean, confidence interval (CI), standard deviation (SD), standard error (SE), median, inter-quartile ranges (IQR) and normality tests (Kolmogorov–Smirnov, Shapiro–Wilk) were done with Excel manually or with add-in modules: Data Analysis Tools and Analyse-it® software, v. 1.73 (Analyse-it Software, Ltd). The first order rates of CL-20 degradation were found by linear regression of ln-transformed experimental data with the LINEST Excel function which uses the ''least squares'' method to calculate regression coefficients and provides their confidence intervals and *F*-statistics.

The number of analytical replicates in this study was chosen to meet specific requirements of kinetic measurements. It is known that variability of biokinetic data most critically depends on the uncertainty of initial conditions, e.g. what is the exact amount of added degradable compound and how evenly is it distributed between replicate tubes and how long is growth lag-phase which depends on the physiological state of microorganisms in soil before substrate addition or cells used as inoculum in batch culture. To minimize this source of errors we used five replicates at the start of the incubation experiment and then proceeded with two replicates at later stages. Besides, we repeated the batch experiments (i.e. ran them twice under each condition) to make sure that the temporal profile was the same with different inocula. Only one (typically the second) curve was demonstrated below in the Results section.

Results

Statistical characterization of the analytical methods used

Figure 1 and Table 1 show results of preliminary experiments on statistical evaluation of the analytical technique developed to quantify residual content of CL-20 in soil. In 41 samples of soil amended with the same amount of CL-20 and incubated 1–2 days without addition of co-substrate, the propellant was not degraded and the observed variation was attributed solely to analytical error. The distribution followed the normal pattern, as could be seen from the frequency histogram (Fig. 1A) and normal plot (Fig. 1B), which was practically linear quantile–quantile plot against standard normal distribution. The Kolmogorov–Smirnov parameter confirmed that null hypothesis about normality could not be rejected. Therefore we can use known statistical formula to calculate the confidence interval for a given number of replicates:

$$\bar{x} \pm z_{\alpha/2} \frac{\sigma}{\sqrt{n}}$$

where $\bar{x}$ is sample mean, n is sample size, σ is population standard deviation, and $z_{\alpha/2}$ is tabulated value of Gaussian integral for a given significance level α.

We assumed that sample standard deviation at $n = 41$ was close to the σ parameter and found the 95% confidence interval ($CI_{0.95}$) for our sampling scheme. With two replicates ($n = 2$), $CI_{0.95}$ was ±2.24 mg l^{-1}, with three and five replicates it dropped respectively to ±1.83 and ±1.48 mg l^{-1}. All found $CI_{0.95}$ values were rather narrow and acceptable including data calculated as mean of two replicates.

The most essential conditions to preserve low SD were (i) soil homogenization by mixing and sieving, (ii) the uniform distribution of CL-20 as stable sonicated suspension passed through paper filter, and (iii) analysis of the entire sacrificed tube rather than repetitive subsampling from one single batch flask. The last point was especially important. When we made repetitive 1 ml subsampling ($n = 20$) from one and the same flask containing 100 ml of soil suspension, the mean was the same as in experiment with sacrificed vials, 53.67 mg l-1 but standard deviation increased five times from 1.6 to 8.03 mg l^{-1}.

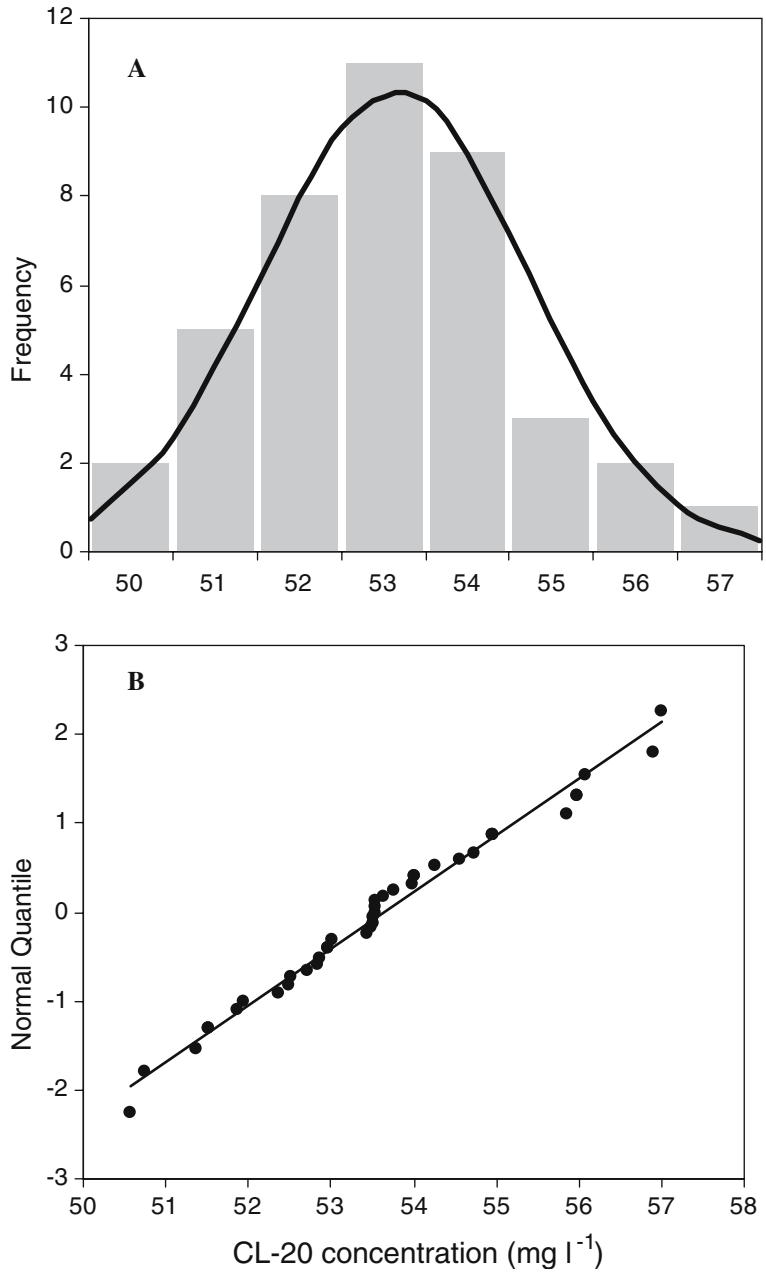


Fig. 1 Test for normal distribution of analytical data including frequency histogram (top) and normal plot (bottom). The set of 41 soil samples was analyzed for CL-20 content as described in the Method section. Kolmogorov–Smirnov normality test was performed with a null hypothesis that distribution is normal. The null hypothesis was not rejected (K–S coefficient =0.5896, *P*-value >0.15). Other parameters are shown in Table 1

CL-20 degradation in soil

We have found very slow degradation of CL-20 in sterile soil or sterile nutrient solutions. The decay rates was about 3% per month or 0.001 day^{-1}. The recovery of the added propellant was 100 ± 5% indicating a negligible amount of irreversible binding of CL-20 to soil particles. Even lower abiotic degradation was found earlier in similar NJ forest soil (Strigul et al. 2006) in contrast to intensive spontaneous decay in the China Lake soil (Crocker et al. 2005). The main difference between NJ forest soil and China Lake soil was pH-value: our soil was slightly acidic (pH 4.9) while China Lake soil was alkaline (pH 8.4) and therefore provided conditions for intensive alkaline hydrolysis of CL-20 (Balakrishnan et al. 2003).

Table 1 Statistical parameters characterizing analytical method

Parameter	Value	
Number of samples, *n*	41	
Mean	53.645	
95% CI	53.149–54.141	
Variance	2.4738	
SD	1.5728	
SE	0.2456	
CV	3%	
Median	53.528	
97.2% CI	52.973–54.013	
Kolmogorov–Smirnov test	0.5896	$P > 0.15$
Skewness	0.2188	0.5348
Kurtosis	–0.2787	0.8150

The non-sterile unamended soil under oxic conditions was also inactive and all tested co-substrates significantly accelerated CL-20 decomposition (Fig. 2). The greatest increase in degradation was observed with succinate and pyruvate. With other C-compounds, the degradation rate declined in the following order: acetate > glucose > starch. The yeast extract (YE), which is source of C and N, also had significant stimulatory effect, although it was not highest.

Under anoxic conditions CL-20 became more accessible even without auxiliary C-substrates: about 25% of added xenobiotic was degraded after 2 weeks (Fig. 3). Addition of succinate accelerated anaerobic biodegradation even further. However, the highest stimulation was achieved by succinate under oxic conditions. Mineral nitrogen did not affect significantly CL-20 decomposition in the original soil because of its high N-content, e.g. the degradation dynamics with succinate as co-substrate was only slightly

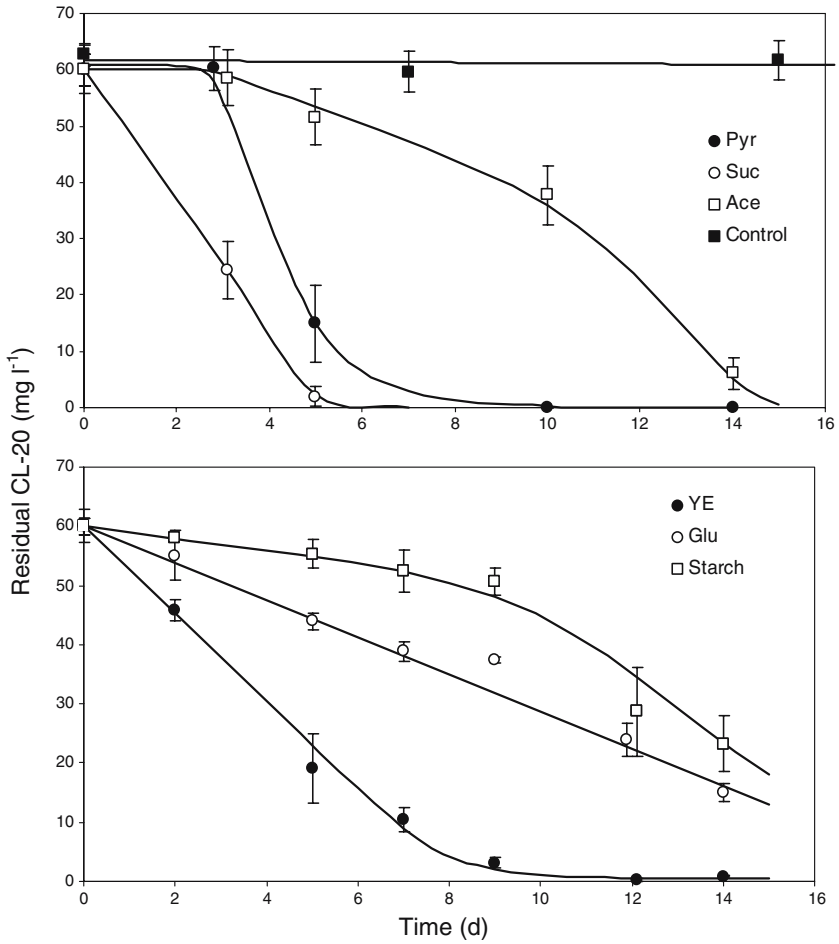


Fig. 2 Effect of auxiliary substrates on the rate of CL-20 degradation in the soil. Dynamics of residual CL-20 concentration was followed in the soil samples amended with mineral nitrogen and various organic substrates (see legend). Control stands for autoclaved soil without co-substrates. The vertical bar here and below shows sample SD

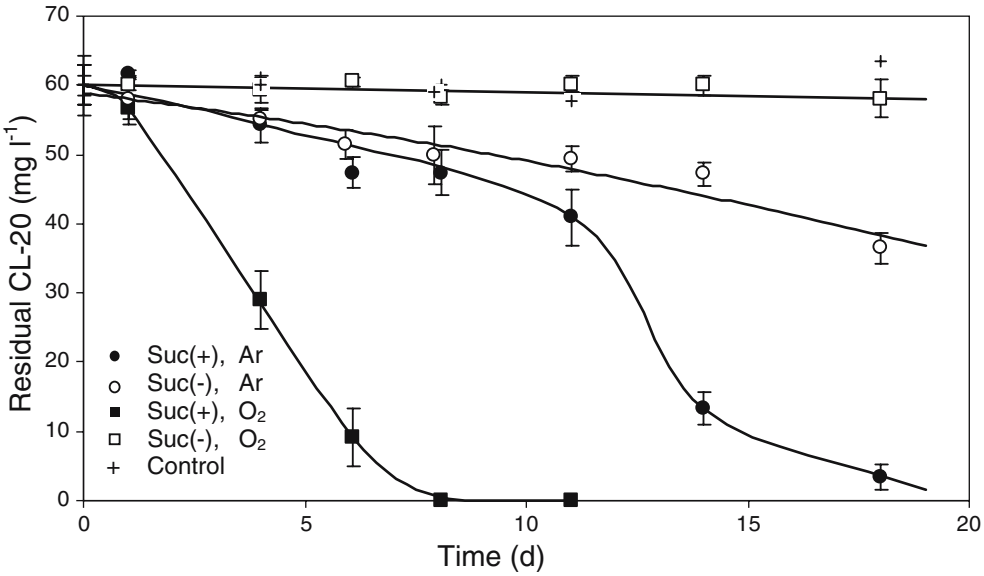


Fig. 3 CL-20 decomposition dynamics in soil under oxic and anoxic conditions. CL-20 was added to soil in combination with succinate (Suc(+), filled circles and squares) or without auxiliary substrate (Suc(–), open circles and squares) and incubated under air (O_2, squares) or argon (Ar, circles) in headspace. No mineral nitrogen was added. Control vials (+), contained CL-20 in sterile mineral solution instead of soil

higher (<20%) when supplemented with N (Fig. 2, open circles) than in soil without added N (Fig. 3, solid circles).

CL-20 decomposition in serial soil dilutions

In the N-free succinate-mineral medium (Fig. 4 top), CL-20 was degraded only in the first dilution, the degradation rate being constant until full exhaustion of xenobiotic. The linear dynamics of residual CL-20 indicates zero cell growth, with the degradation probably being performed by residual enzymatic activity of non-growing cells. In the second and following dilutions, CL-20 degradation was zero.

In the succinate medium supplemented with N (Fig. 4, bottom), CL-20 degradation took place up to the 6th dilution. Generally, the higher the dilution and the smaller the initial amount of degrading microorganisms, the longer the lag phase preceding intensive degradation. The CL-20 degradation dynamic turned non-linear with progressive acceleration, indicating growth of microbial degraders in each positive dilution tube.

Isolation of microorganisms degrading CL-20

Plating from soil gave abundant bacterial and fungal growth on succinate N(+) medium. Almost half (44%) from the 30 randomly tested colonies were able to decompose CL-20. None of them displayed any significant growth acceleration in the presence of CL-20. Based on microscopic examination, culturing and 16s rDNA gene sequence, six isolates were identified as representatives of five genera of organisms widely distributed in polluted environments (Table 2). The highest growth rate and degradation activity was displayed by *Pseudomonas* sp. MS-P.

CL-20 degradation in a pure culture of *Pseudomonas* sp. MS-P

Figure 5 shows growth and concomitant CL-20 disappearance in the batch culture as dependent on initial succinate concentrations. The cell suspension grew exponentially the first 3 days until the full depletion of succinate, the peaks of cell mass values being roughly proportional to the initial succinate concentration. After depletion of succinate and set-up of starvation phase, the cell

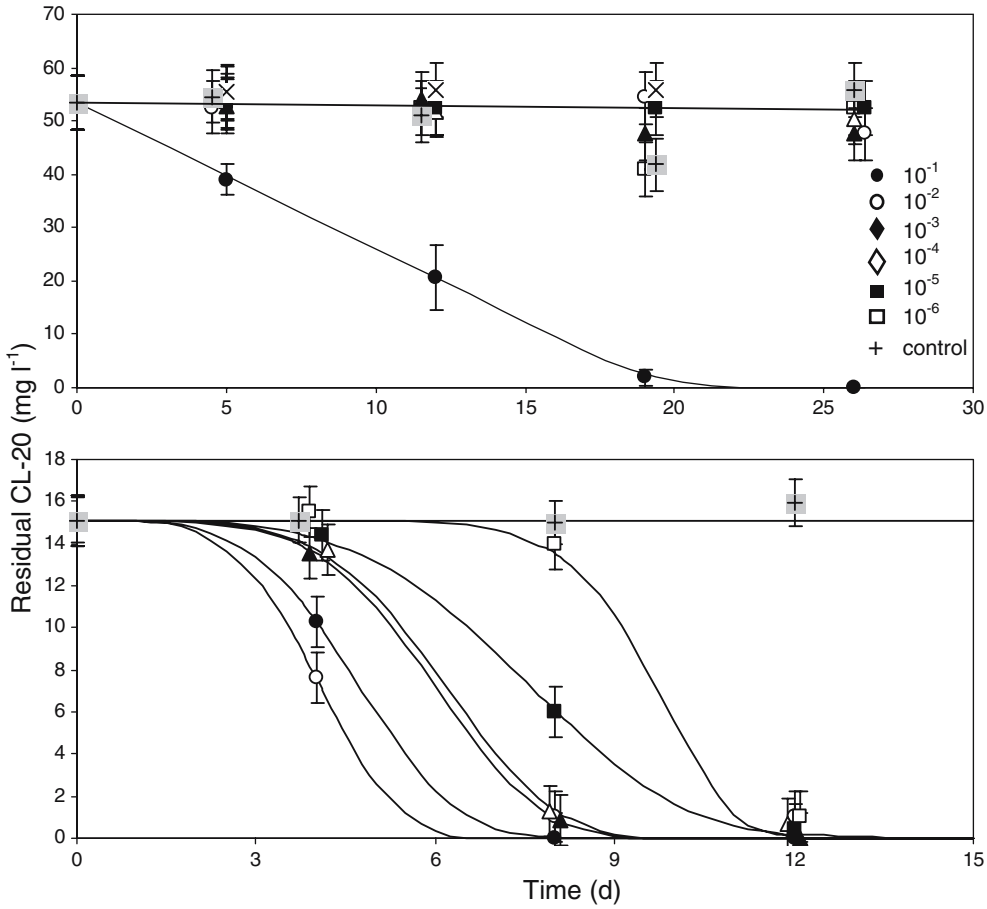


Fig. 4 CL-20 decomposition dynamics in serial dilutions of soil suspension. Top: medium contains CL-20 + succinate-mineral solution without N. Bottom: the same with N added as $(NH_4)_2SO_4$

density rapidly declined, this being a characteristic of members of genus *Pseudomonas* (Panikov 1994). The following progressive decrease in the death rate was due to cell transition to partial dormancy (Panikov 1995). The dynamics of CL-20 degradation was adequately approximated by exponential curves indicating first order decomposition kinetics. Irrespective of the bacterial growth phase (growth, decline) the degradation rate was proportional to the instant residual concentration of CL-20, although the steepness of decline was higher at higher initial succinate concentration.

To find out whether CL-20 transformation is coupled tightly to succinate uptake, we performed the final experiment (Fig. 6): the cells were grown without CL-20 until complete uptake of succinate and then CL-20 was added either alone or in combination with succinate at concentration 10 and 20 g l^{-1}. It turned out that non-adapted and starving cells do degrade CL-20 although at slower rate than in growing cultures. Therefore we can conclude that pre-adaptation and presence of succinate is not absolutely necessary for co-metabolic degradation of CL-20. At the highest succinate concentration (20 g l^{-1}), cell growth decelerated on the second day of cultivation probably because of oxygen limitation or product inhibition (Fig. 6). As a result, the doubling of C-substrate from 10 to 20 g l^{-1} did not result in doubling of cell yield but instead caused delay in starvation and extended the stationary phase of the batch culture.

Table 2 Microbial degraders of CL-20

Organism, strain	Cell morphology	CL-20 degradation rate, day^{-1} [a]	BLAST search for related phylotypes (similarity)	The confidence threshold, % (Cole et al. 2003)
Pseudomonas sp. MS-P	Rod-shaped gram-negative motile asporogenous bacteria, 0.8–2.0 × 0.3–0.7 μm	0.06–0.11	*Pseudomonas putida* (99%)	*Pseudomonas*, 100
Rhodococcus sp. MS-1	Pleomorphic (irregular rods-to-coccoid) non-motile and asporogenous gram-positive aerobic bacteria. Produces abundant extracellular slime	0.01–0.09	*Rhodococcus opacus* (97%) *R. equi* (97%) *R. erythropolis* (97%)	*Rhodococcus*, 100
Rhodococcus sp. MS-6	The same but no slime production	0.05–0.07	*R. erythropolis* (99%)	*Rhodococcus*, 100
Ochrobactrum sp. MS-8	Motile, clock-wise rotating rods with rounded ends, 0.8–1.8 × 0.5–1.0 μm; aerobic asporogenous gram-negative or gram-variable (in old culture)	0.07–0.09	*Ochrobacrum* sp. AY576683 (99%) *Ochrobacrtum* sp. AF229879 (99%)	*Brucellaceae*, 100 *Ochrobactrum*, 74
Mycobacterium sp. MS-11	Pleomorphic (Y- and V-shaped, rods and ovals) non-motile and asporogenous cells 0.7–2.2 × 0.3–0.9 μm; gram-positive aerobic; form aggregates and biofilms in shake flasks	0.02–0.04	*Mycobacterium fortuitum* (99%) *M. porcinum* (99%)	*Mycobacterium*, 100
Ralstonia sp. MS-10	Motile and asporogenous rod-shaped cells 0.6–1.4 × 0.3–0.6 μm; gram-negative aerobic	0.07–0.10	*Ralstonia pickettii* (99%)	*Ralstonia*, 100

[a] The initial CL-20 and Na-succinate concentration were respectively 10 mg l^{-1} and 2 g l^{-1}, analysis of residual CL-20 was made on days 7 and 14, other conditions see in Materials and methods. The specific degradation rate of CL-20 was calculated from eq. 1 for two replicate vials

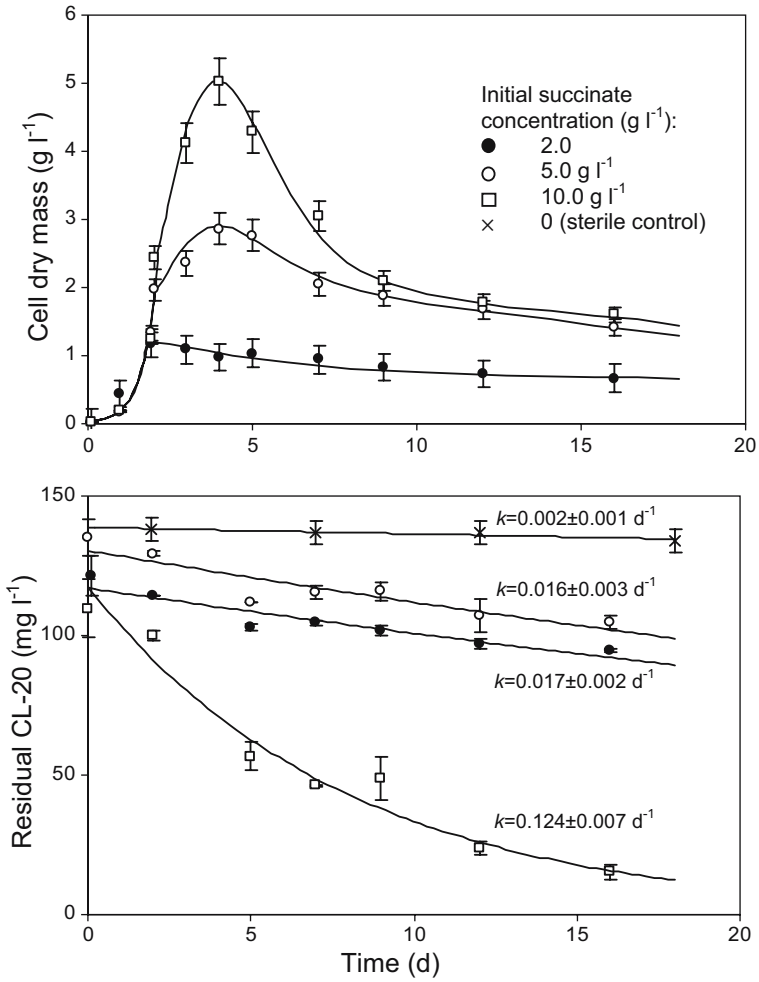


Fig. 5 Bacterial growth (top) and corresponding CL-20 decomposition dynamics (bottom) in the batch culture of *Pseudomonas* sp. MS-P as dependent on initial concentrations of succinate in the medium. Data points on residual CL-20 were least-square fit to exponential curves, the number indicate first order rate constant ± standard error

Discussion

Degradation kinetics

In our study, we confirmed earlier findings (Trott et al. 2003) indicating that biodegradation of CL-20 is accelerated under anoxic conditions while the aerobic process strictly depends on the presence of organic co-substrate (Figs. 2 and 3). Succinate and pyruvate were better than other tested compounds including complex growth substrates like yeast extract.

Our main conclusion drawn from this study (and what has been overlooked by other researchers) is that CL-20 is degraded via *co-metabolic reactions* uncoupled from degrader growth. Usually co-metabolism is defined as 'gratuitous metabolic transformation of a substance by a microorganism growing on another substrate; the co-metabolized substance is not incorporated into cells and does not serve as source of energy' (Atlas 1997).

The most convincing evidence for co-metabolism came from kinetic analysis of the batch growth (Figs. 5 and 6). The observed curves of residual CL-20 concentrations (s) were accurately approximated with the exponential functions implying that degradation follows simple first order kinetics:

$$\frac{ds}{dt} = -ks; \quad s = s_0 e^{-kt} \tag{1}$$

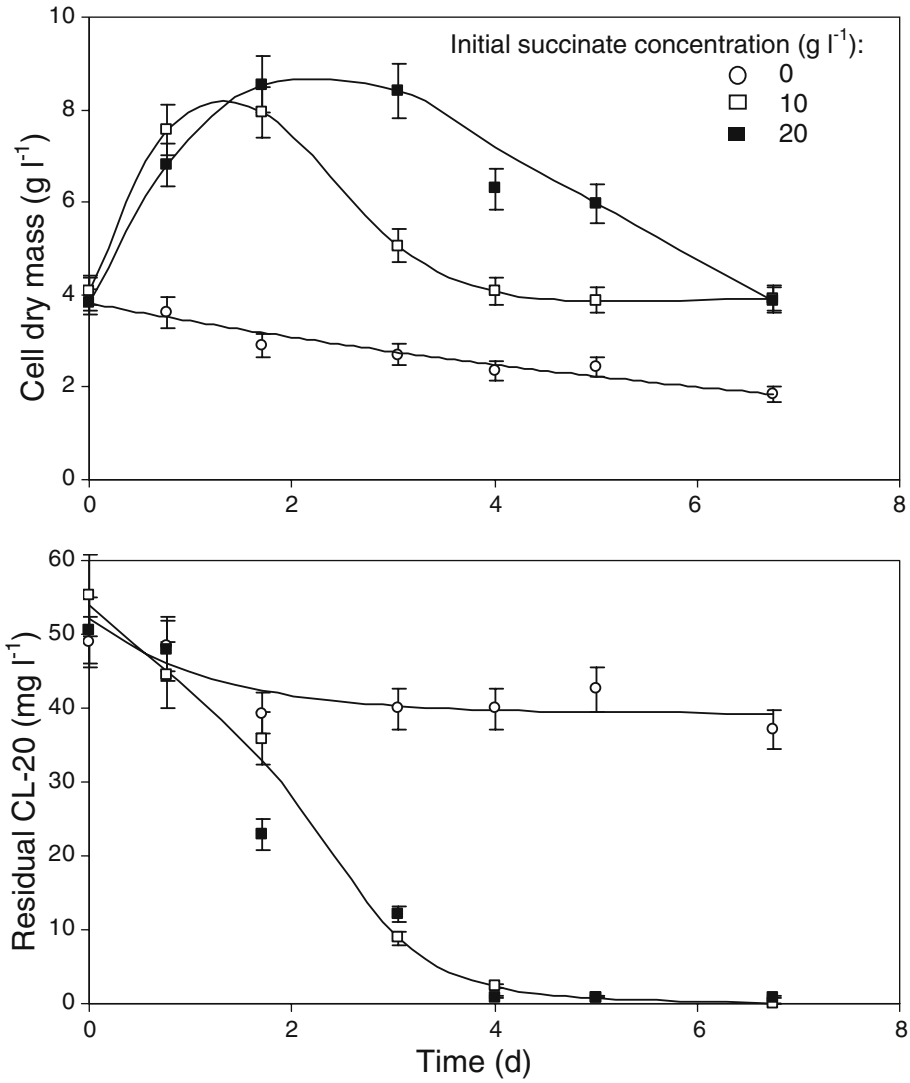


Fig. 6 CL-20 decomposition by starving and growing bacteria *Pseudomonas* sp. MS-P. At time zero CL-20 was added to stationary culture of bacteria without succinate (○) or in combination with 10 g l^{-1} (□) or 20 g l^{-1} of Na-succinate (■). The top panel shows cell mass dynamics and the bottom panel displays the corresponding dynamics of residual CL-20

where t is time, s_0 is the initial CL-20 concentration ($s = s_0$ when $t = 0$) and k is constant.

The dynamics of batch culture with cell growth and subsequent starvation decline (Fig. 5 top) could be fit only to much more complicated kinetic models, the minimal version being represented by a set of three non-linear differential equations simulating 'feast-to-famine' transition (Panikov 1995):

$$\begin{aligned} \frac{dx}{dt} &= (\mu - ar)x, \quad \mu = Y(q_s - mr) \\ \frac{ds_{suc}}{dt} &= -q_s x, \quad q_s = Q_s r \frac{s_{suc}}{K_s + s_{suc}} \\ \frac{dr}{dt} &= (\mu + ar)\left(\frac{s_{suc}}{K_r + s_{suc}} - r\right) \end{aligned} \tag{2}$$

where x is cell mass, s_{suc} is co-substrate concentration, r is intracellular ribosome content, μ

and q_s are specific growth and substrate uptake rates respectively, a is turnover rate and m is maintenance coefficient, Y, Q_s, K_s and K_r are kinetic constants. An account of ribosome content (which correlates with content of enzymes catalyzing an entire primary metabolism) is absolutely necessary to describe lag-phase and acclimation to starvation conditions during the stationary phase as well as other changes in the 'quality' of cells when they proceed through batch cycle. Without going into further detail, we conclude that an adequate kinetic model of cell growth would be much more complex and different from simpler biodegradation kinetics.

However, the apparent simplicity of eq. 1 describing CL-20 degradation should not mislead us. Contrary to abiotic reactions, the rate constant, k in eq. (1) is not a fundamental constant but varies depending on initial succinate concentration and cell density in batch culture. The initial enzymatic degradation of CL-20 should follow as a minimum the Michaelis–Menten kinetics with reduction to first-order case under condition $s \ll K_m$:

$$\frac{ds}{dt} = -V\frac{s}{K_m + s} \sim -\left(\frac{V}{K_m}\right)s = -ks, \quad k = \frac{V}{K_m} \tag{3}$$

where K_m is the Michaelis constant and V is the maximal rate of enzymatic reaction which can be represented as a product of the catalytic constant k_{cat} and the concentration of enzyme active centers e_0: $V = k_{cat} \times e_0$. At low substrate concentration, the rate of enzymatic reaction proceeds as first-order with apparent constant k expressed via Michaelis parameters:

$$k = V/K_m = k_{cat} \times e_0/K_m \tag{4}$$

The restrictive condition $s \ll K_m$ is not unusual, because of the low solubility of CL-20; even if we use *concentrated suspensions* of CL-20, the actual s-value (concentration of soluble CL-20 around the enzyme's active centers) must be well below typical K_m values ~10^{-3} M for the majority of dehydrogenases.

Tentative mechanism of anoxia effects

The accelerated CL-20 degradation under anoxic conditions also agrees with the co-metabolism model, if we take into account published data on enzymatic degradation of CL-20. Most enzymes used for degradation are dehydrogenases which start degradation by transferring $\bar{e}$/H from NADH to CL-20 (Bhushan et al. 2004, 2004a, 2005). It is well known that this reduction reaction is inhibited by O_2 because oxygen is a competing electron acceptor. Further acceleration by succinate addition was due to increase in the flux of NADH and the total amount of constitutive dehydrogenases due to growth of soil microorganisms. Under anoxic conditions, microbial growth supported by succinate fermentation should be less efficient than aerobic respiration producing less cells and enzymes including enzymes responsible for co-metabolic degradation.

Effect of N and use of CL-20 as the N-source

In our experiments with undiluted soil slurry, N addition did not affect CL-20 degradation, while in soil dilutions and bacterial cultures, the mineral N turned out to be crucially needed to degrade CL-20. There is no contradiction in these observations because original soil contained significant reserves of N, 9 mg g^{-1}. Serial 10-fold dilutions of soil were done with N-free medium containing the same concentration of C-source; that caused a rapid rise in C:N ratio from 3.5:1 in the undiluted soil slurry to 22:1 in the first dilution, 220:1 in the second dilution and so forth[1]. Therefore the second decimal dilution already looks inappropriate for growth if it is not supported by N_2 fixation or other N-sources. It is interesting that in the presence of CL-20, N_2 fixation seems to be suppressed, and under N-starvation, CL-20 was not accessible. Probably some minimal N-priming was required to initiate microbial growth and accumulate enzymes and reducing power (NADH) for denitration of CL-20.

[1] To calculate C:N ratio, we used direct data on soil weight in the first dilution and assumed that 10% of soil N is immediately available for bacterial growth, the rest becoming available after a long-term mobilization process.

Contrary to our results, Trott et al. (2003) grew *Agrobacterium* sp. on succinate-mineral medium containing CL-20 as a sole source of N. The linear relationship was found between cell protein and the amount of nitrogen added as CL-20. Degradation stoichiometry was found to be 3 mol of assimilated nitrogen per mol of CL-20 and growth stoichiometry was 213 g of cell protein per mol of CL-20.

The reason for discrepancy between our results and published data remain unclear. We used similar techniques with only one essential exception: we used ultrasound dispersion while Trott et al. (2003) applied Tween to stabilize aquatic suspension of CL-20. It is possible that this mild detergent may have played the role of an additional available substrate or neutralized the toxicity of some degradation products, e.g. nitrite. Further studies are needed to clarify this point.

From an environmental perspective, the use of N derived from degraded CL-20 is of minor significance. First, even heavily contaminated soil should still preserve a large amount of endogenous soil N that is essentially more accessible than N derived from pollutant CL-20. It is extremely unlikely that growth of CL-20 degraders in situ will be N-limited. Second, the bottleneck of biodegradation under any circumstances continues to be the cleavage of the heterocyclic molecule with dehydrogenase or a similar enzyme, not utilization of N-compounds (nitrite, ammonium, N_2O). Third, all members of a soil community share the N derived from CL-20 because it remains in the soil extracellular space and because *there is no specialized metabolic way* to assimilate these forms of N. Therefore even under improbable conditions of N-deficiency, the degraders do not have competitive advantage over other non-degrading organisms.

CL-20 degraders

Almost half (44%) of randomly selected colonies grown on succinate had the ability to degrade CL-20. Therefore we can conclude that this metabolic quality is widespread among microorganisms inhabiting soils and probably other natural environments.

All active microorganisms isolated in the present study belong to the category of active degraders of various xenobiotics. The longest list of reports is available for *Pseudomonas putida* and *Rhodococcus erythropolis*, which are frequently found as dominant in enrichments degrading hydrocarbons from various polluted environments (Finnerty 1992; Thomassin-Lacroix et al. 2001; Van Hamme et al. 2003; Wackett 2003). Non-pathogenic forms of *Mycobacterium* belong to the same taxonomic group of actinobacteria as *Rhodococcus*. They share such common features as pleomorphic appearance (filaments–rod–cocci transition) and ability to degrade hydrophobic pollutants even in the presence of more readily available carbon sources. Bacteria of the genus *Ralstonia* and *Ochrobactrum* were also found to have active degradation enzymes (Fishman et al. 2004) and to be able to degrade a wide range of chlorinated and nitrated xenobiotics (Muller et al. 1998, 2002; Smejkal et al. 2003).

The bioremediation perspective

As a chemical that is persistent and highly toxic to animals and humans, CL-20 could be an extremely hazardous environmental pollutant. The conventional remediation approach based on isolation and in situ application of a single 'active' microbial degrader could easily fail for a number of reasons: inadequate environmental conditions for industrial strain, spatial heterogeneity of natural habitat, predation and antagonistic suppression of the released bacteria, etc. And the most important risk-factor for failure would be the co-metabolic nature of CL-20 biodegradation, which excludes any kind of competitive advantage of degraders over other members of the microbial community in polluted soil. Instead of searching for a single species of degrader (or in addition to this goal), we should rely more on the in situ stimulation of the indigenous community of the polluted environment by using appropriate auxiliary substrates. Such a solution looks essentially less expensive and is sounder from the perspective of microbial ecology.

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