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Degradation of 2,4,6-trichlorophenol by a specialized organism and by indigenous soil microflora: bioaugmentation and self-remediability for soil restoration

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V. ANDREONI, G. BAGGI, M. COLOMBO, L. CAVALCA, M. ZANGROSSI AND S. BERNASCONI. 1998. A selected mixed culture and a strain of *Alcaligenes eutrophus* TCP were able to totally degrade 2,4,6-TCP with stoichiometric release of Cl^-. In cultures of *Alc. eutrophus* TCP, a dioxygenated dichlorinated metabolite was detected after 48 h of incubation. Experiments conducted with soil microcosms gave evidence that: the degradative process had a biotic nature and was accompanied by microbial growth; the soil used presented an intrinsic degradative capacity versus 2,4,6-TCP; the specialized organism used as inoculum was effective in degrading 2,4,6-TCP in a short time. These results could be utilized for the adoption of appropriate remediation techniques for contaminated soil.

INTRODUCTION

Chlorophenols (CPs), widely used as preservatives for different goods, anti-mildew agents, disinfectants and fungicides (Rao 1978; Sittig 1981), can be formed during the chlorination of waste waters, and are often by-products of the breakdown of pesticides and chlorinated aromatic compounds (Pritchard *et al*. 1987). These substances, which are carcinogenic and can condense to chlorodibenzodioxins, are extremely toxic and hazardous to the environment (Rochkind *et al*. 1986). They are included in the US Environmental Agency Priority Pollutant Lists (Anon. 1987). In particular, 2,4,6-trichlorophenol (2,4,6-TCP), which is a precursor for the synthesis of 2,3,4,6-tetrachlorophenol, pentachlorophenol (PCP) and fungicide prochloraz, is detected in large amounts in the environment as it is derived as an intermediate from the breakdown of prochloraz (Bock *et al*. 1996) and is found as a major component of kraft paper mill effluents (Huynh *et al*. 1985).

The microbial degradation of CPs, which may be abiotically degraded through hydrolysis and photolysis (Hwang *et al*. 1986), has been studied with mixed and pure microbial cultures; under aerobiosis, the metabolic pathway of CPs proceeds through the formation of chlorocatechols for mono- and dichlorophenols, and chlorohydroquinones for highly chlorinated phenols (Steiert and Crawford 1986; Li *et al*. 1991; Kiyohara *et al*. 1992; Wieser *et al*. 1994; Fava *et al*. 1995; Tomasi *et al*. 1995; Bock *et al*. 1996; Koh *et al*. 1997). Under anaerobic conditions, CPs, independently from the substitution grade, are often reductively dechlorinated up to phenol in fresh water sediments and by microbial consortia under sulphate-reducing and methanogenic conditions (Zhang and Wiegel 1990; Häggblom and Young 1990; Turner Togna *et al*. 1995); few pure cultures have been shown to degrade CPs anaerobically (Mohn and Kennedy 1992; Utkin *et al*. 1995; Christiansen and Ahring 1996).

Here, the aerobic degradation of 2,4,6-TCP in batch by a mixed culture and a pure strain of *Alcaligenes eutrophus* TCP is reported. In addition, experiments with soil microcosms were performed to evaluate whether the micro-organism isolated, able to degrade *in vitro* 2,4,6-TCP, should be used as inoculum to remediate soils contaminated by this specific pollutant.

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MATERIALS AND METHODS

Enrichment cultures

A mixed culture able to grow on 2,4,6-TCP supplied as the sole carbon and energy source in a mineral medium (MM) was selected by enrichment techniques using as inoculum activated sludges of a waste-water plant. The composition of

MM was (l^{-1}): Na_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 0·8 g. The pH was adjusted to 7·0, the medium was autoclaved and then 2·5 ml of 1 mol l^{-1} $MgSO_4$, 2·5 ml of 36 mmol l^{-1} $FeSO_4.7H_2O$, and 2·5 ml of a trace element solution, were added (Maniatis *et al*. 1989).

2,4,6-TCP was added to MM medium at the required concentration from a stock solution (5% w/v) in acetone. Other soluble organic substrates (phenol, glutamate etc.) were used as carbon source at 0·02% (w/v).

Isolation and maintenance of 2,4,6-TCP degrading micro-organism

From the selected mixed culture, a microbial strain was isolated in the presence of 200 mg l^{-1} 2,4,6-TCP as the only carbon and energy source. The strain was maintained through subsequent transfers in MM with 300 mg l^{-1} 2,4,6-TCP added and frequently checked for purity by plating in Plate Count Agar (PCA) (Difco).

Phenotypic and molecular characterization of the bacterial strain

The phenotype of the strain was characterized by standardized procedures (Gram stain, mobility, etc) (Doetsch 1981) and by the Biolog GN identification test (Biolog Inc., Hayward, CA, USA).

The characterization at molecular level was performed by amplified ribosomal DNA restriction analysis (ARDRA). The restriction map of the present strain was evaluated in relation to those of *Alc. eutrophus* ATCC 17697 retrieved from GenBank (accession number: m32021) and performed by Webcutter© (Max Heim, 1995, http://www.medkem.gu.se/cutter), *Alc. eutrophus* JMP 134 and *Comamonas acidovorans* DSM 8370. Template DNA (5 μl) prepared by Chelex® 100 (Biorad) boiling lysis of cell suspension (De Lamballerie *et al*. 1992), was used to perform PCR amplification in 50 μl reaction volume. The mix was prepared with polymerase reaction buffer (10 mmol l^{-1} Tris-Cl (pH 8·3; 25 °C); 50 $mmol^{-1}$ KCl; 1·5 mmol l^{-1} $MgCl_2$; 20 μmol l^{-1} (each) dATP, dCTP, dTTP, dGTO; 0·2 μmol l^{-1} each of 16S rRNA universal primer, placed in positions 27 forward and 1495 reverse (Weisburg *et al*. 1991), and 1·25 U of *Taq* polymerase (Promega). PCR amplifications were performed using a thermal cycler (Perkin Elmer GeneAmp PCR System 2400) with the following temperature profile: five cycles of denaturation (1 min at 94 °C), annealing (45 s at 50 °C), and extension (1 min at 72 °C); 35 cycles of denaturation (40 s at 94 °C), annealing (45 s at 55 °C), extension (1 min at 72 °C); and final extension at 72 °C for 7 min. Aliquots (8 μl) of PCR products were digested with 10 U of restriction endonuclease in 10 μl of reaction volumes using the manufacturer's recommended buffer and temperature. The following restriction endonucleases (Pharmacia Biotech) were used: *AluI*, *Eco*RI, *Hha*I, *Hpa*II and *Rsa*I. Restricted DNA was analysed by horizontal electrophoresis in 3% agarose gel (Pharmacia), carried out at 80 V for 3 h with 11 × 13 cm gels (Easy-Cast B2, Owl Scientific Inc., Woburn, MA, USA).

Similarities between each pair of strains were estimated from the proportion of shared restriction fragments to the total number of fragments identified for all strains, and examined using Jaccard's coefficient (Sokal and Michener 1958). A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) using an NTSYS-pc analysis package (Rohlf 1987).

Soil characteristics

The soil for microcosms was kindly provided by CNR-Pisa and, in the last 20 years, had not been treated with pesticides. It was characterized as a lime clay soil with a low organic content (1·6%), CEC (12·4 meq 100 g^{-1}), pH (H_2O) (8·2). The soil contained $1·9 \times 10^7$ colony forming units (cfu) g^{-1} dry soil as enumerated on PCA. Soil pH was not adjusted and additional nutrients were not used. The soil was stored at approximately 5 °C until used.

Soil microcosms

Laboratory microcosms were prepared in triplicate, placing 10 g of air-dried soil into a 50 ml beaker and adjusting the soil moisture content to 60–80% of field capacity with distilled deionized water. The beakers, covered with aluminium foil to minimize water loss and contamination, were conditioned for 5 d at 30 °C before adding 2,4,6-TCP at 250 mg kg^{-1} soil from a stock solution. Each experiment consisted of four sample sets: autoclaved soil (AS), autoclaved soil inoculated with the microbial strain selected (AIS), soil (S), and inoculated soil (IS). For AS and AIS microcosms, the soil was autoclaved three times at 121 °C for 20 min at 24 h intervals. For S and IS microcosms, the soil was used directly.

For microcosm inoculation, the microbial strain was grown at 30 °C on a shaker in MM with added 2,4,6-TCP. When the culture reached the late logarithmic phase of growth, the cells were collected by centrifugation at 12 000 *g* for 20 min at 4 °C, washed with 0·1 mol phosphate buffer (pH 7·0) and suspended in the same buffer to obtain a cell suspension of approximately $8·0 \times 10^7$–$4·0 \times 10^9$ cfu ml^{-1}. When required, 1 ml of cell suspension at low or high density was added as inoculum to each microcosm and mixed thoroughly with the soil.

The beakers were incubated in the dark at 30 °C, checking the moisture content every 7 d. In some cases, 500 mg 2,4,6-TCP kg^{-1} soil were added after 2 d of incubation.

For determining 2,4,6-TCP loss and bacterial growth, soil

microcosms were analysed on days 0, 1, 2 and 5. 2,4,6-TCP concentration was determined as the quantity of compound present that was water-extractable (WSF) per unit of the soil according to Dasappa and Loehr (1991). The beaker contents were placed in a 300 ml sterile flask containing 200 ml of sterile deionized water, and mixed for 1 h on a rotary shaker (250 *g*) at 30 °C. After 30 min sedimentation, an aliquot of the supernatant fluid was diluted (1 : 10) in tubes containing 9 ml of sterile water, and all dilutions were then plated in duplicate into PCA. The inoculated plates were incubated at 30 °C for 5 d and then counted. The remaining supernatant fluid, after centrifuging at 10 000 *g* for 30 min, was filtered (0·45 μm) and stored at 4 °C.

Isolation and characterization of the 2,4,6-TCP metabolite in batch experiments

Alcaligenes eutrophus TCP was incubated at 30 °C in Erlenmeyer flasks containing MM with 200 mg l^{-1} 2,4,6-TCP added. After 48 h of incubation, the contents of the flasks were collected and centrifuged at 10 000 *g*. The supernatant fluid was extracted with ethyl acetate; the organic layer was dried over $MgSO_4$ and evaporated to dryness in a rotary evaporator. The residue was analysed by gas chromatography–mass spectrometry (GC–MS).

Analytical procedures

The decrease of 2,4,6-TCP content in batch experiments was determined by high performance liquid chromatography (HPLC), directly injecting the supernatant fluid obtained from samples of the cultures previously centrifuged at 10000 *g* for 20 min to separate the cells and filtered (0·45 μm).

Reversed-phase HPLC was carried out using a Jasco instrument (LG 980-02 Ternary gradient Unit) with UV–VIS detector (UV-975 Intelligent Jasco, Tokyo, Japan). A Spherisorb ODS 2-04-0525 RP 18 column was used for quantification of 2,4,6-TCP in batch cultures and a Merck Lichrosorb RP8 column in microcosm experiments.

Gas chromatographic analyses were carried out using a Dani (Monza, Italy) 86.10 gas-chromatograph, linked to a Hewlett-Packard (Palo Alto, CA, USA) HP 3396 A integrator, equipped with a fused silica capillary column WCOT-CP-Sil 5-CB Chrompack (4330 EA, Middleburg, The Netherlands) (25 m × 0·32 mm internal diameter, film thickness 0·11 μm), carrier H_2 (0·5 kg cm^{-2}), split flow 90 ml min^{-1}, injection temperature 150 °C, detection temperature 200 °C, initial oven temperature 70 °C (4 min), temperature increase 10 °C min^{-1}, final isotherm 130 °C.

GC-MS data were obtained with a Dani 3800 gas chromatograph connected to a VG-7070 EQ mass spectrometer (the same column was used).

Chloride anion release was determined turbidimetrically at 460 nm by the mercury (II) thiocyanate method (Florence and Farrar 1971).

Chemicals

2,4,6-TCP was from Aldrich Chemical Co. The other chemicals were of the highest grade commercially available.

RESULTS

Degradation of 2,4,6-TCP by mixed culture

The selected enrichment culture rapidly degraded 200 mg l^{-1} 2,4,6-TCP with stoichiometric release of chloride when supplied both as the only carbon source and in the presence of other organic substrates (phenol, benzoate, glutamate 0·02%). The 2,4,6-TCP degradation rate was markedly higher in the presence of phenol or glutamate (Fig. 1).

Isolation and identification of a 2,4,6-TCP-degrading micro-organism

From the selected mixed culture, a Gram-negative bacterium capable of growing on 2,4,6-TCP as the sole carbon and energy source was isolated. The strain was first identified as belonging to *Alcaligenes eutrophus* with a similarity of 62%, according to the biolog GN test. Its molecular characterization was consistent with this phenotypic identification. The dendrogram from the similarity matrix built on ARDRA profiles (data not shown) grouped the micro-organism with *Alc. eutrophus* strains (Fig. 2). The sequence analysis of the 16S rRNA gene should confirm this phylogenetic position.

Degradation of 2,4,6-TCP in batch cultures and in soil microcosms

Alcaligenes eutrophus TCP was able to degrade 300 mg l^{-1} 2,4,6-TCP with stoichiometric release of chloride in 48 h; 500 mg l^{-1} 2,4,6-TCP was totally degraded in 96 h. Higher 2,4,6-TCP concentrations (700 mg l^{-1}) were not degraded at all. Five additions of 2,4,6-TCP were completely degraded in 14 d with stoichiometric release of chloride (Fig. 3).

The strain also grew on phenol (0·5%) but did not utilize for growth other CPs tested (2-, 3- and 4- chlorophenol, 2,4-, 2,6-, 3,4- and 3,5-dichlorophenol, 2,3,5-, 2,3,6- and 2,4,5-trichlorophenol).

A metabolite was isolated from *Alc. eutrophus* TCP cultures, after 48 h of incubation, whose mass spectrum gave PM 178, corresponding to the compound $C_6H_4Cl_2O_2$ (Fig. 4), deriving from the substitution of a chlorine atom with a hydroxyl group.

2,4,6-TCP was completely degraded in all monitored microcosms, except in the autoclaved soil (AS) used as

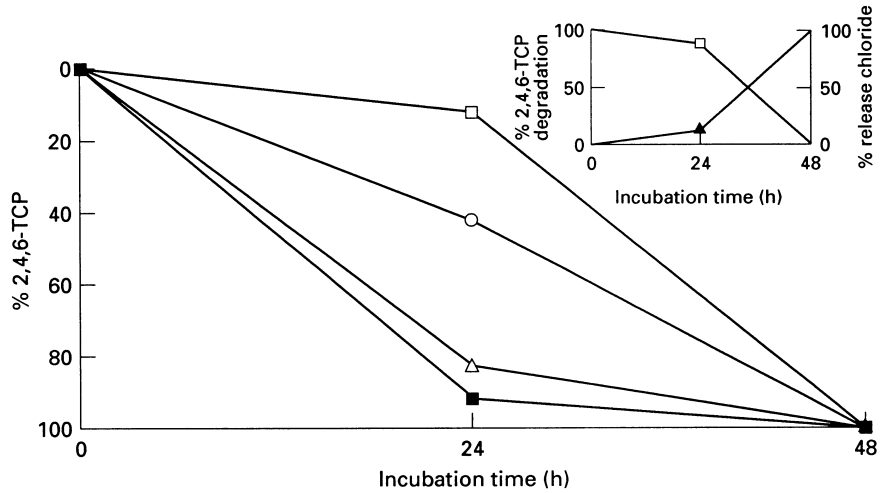

Fig. 1 2,4,6-TCP degradation rate by the mixed culture. □ 2,4,6-TCP 200 mg l^{-1}; △, 2,4,6-TCP 200 mg l^{-1} + phenol 200 mg l^{-1}; ○, 2,4,6-TCP 200 mg l^{-1} + Na benzoate 200 mg l^{-1}; ■, 2,4,6-TCP 200 mg l^{-1} + glutamate 200 mg l^{-1}; ▲, chloride

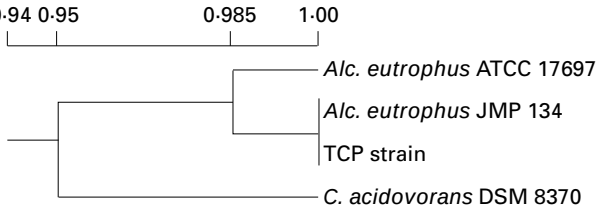

Fig. 2 Phylogeny of *Alcaligenes eutrophus* strains ATCC 17697 and JMP 134, *Comamonas acidovorans* DSM 8370 and the studied TCP strain based on 16S rDNA genotypes characterized by PCR-RFLP analysis. The upper axis indicates genetic similarity

control. The degradation rate was higher when the soil microcosm was inoculated with *Alc. eutrophus* TCP (IS) (100% in 24 h). A further addition of 500 mg kg^{-1} 2,4,6-TCP to the microcosms on the second day of incubation was totally degraded in 72 h in the inoculated microcosm (IS). The control, consisting of autoclaved soil inoculated with *Alc. eutrophus* cells (AIS), gave the same rate of degradation observed in the soil microcosm (S) (Fig. 5)

Bacterial growth was measured in inoculated microcosms at different incubation times. When the soil microcosms (IS, AIS) were inoculated with high cell density, growth reached the same amount. However, the growth was higher in the microcosm inoculated (AIS) with lower initial cell density; from an initial density of $6{\cdot}7 \times 10^6$ cfu g^{-1} soil the population grew to $1{\cdot}9 \times 10^{10}$ cfu g^{-1} soil after 5 d of incubation (Table 1). *Alcaligenes eutrophus* TCP was found to persist at high charge in autoclaved soil (AIS) up to 10 d from inoculation (around 10^{10} cfu g^{-1} soil).

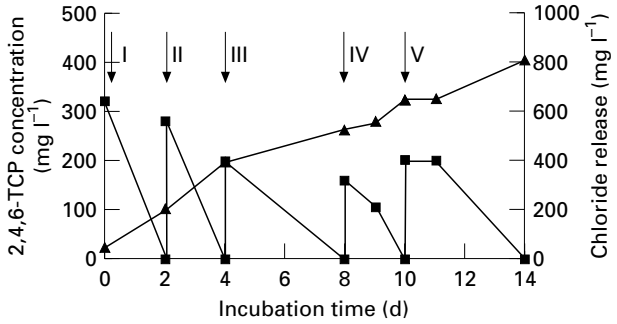

Fig. 3 2,4,6-TCP degradation rate by *Alcaligenes eutrophus* TCP. ■, 2,4,6-TCP concentration; ▲, chloride concentration. Arrows indicate successive 2,4,6-TCP additions (I, II = 300 mg l^{-1}; III, IV, V = 200 mg l^{-1})

DISCUSSION

A bacterial strain able to grow on 2,4,6-TCP as the unique carbon source was isolated from a selected enrichment culture. The isolate, characterized on the basis of phenotypic and molecular features, was classified as *Alc. eutrophus* TCP. An *Alc. eutrophus* JMP 134 (pJP4), originally described for its ability to grow on 2,4 D and other xenobiotics, has been reported to be capable of degrading 2,4,6-TCP without detection of metabolic intermediates (Clément *et al.* 1995). This degradative capacity seemed not to be related to pJ4. This strain was also able to degrade CPs when the cells grew on bleached kraft mill effluent (Valenzuela *et al.* 1997).

The experiments performed in batch show that 2,4,6-TCP is completely degraded with stoichiometric release of chloride anions by the *Alc. eutrophus* TCP strain. The transient for-

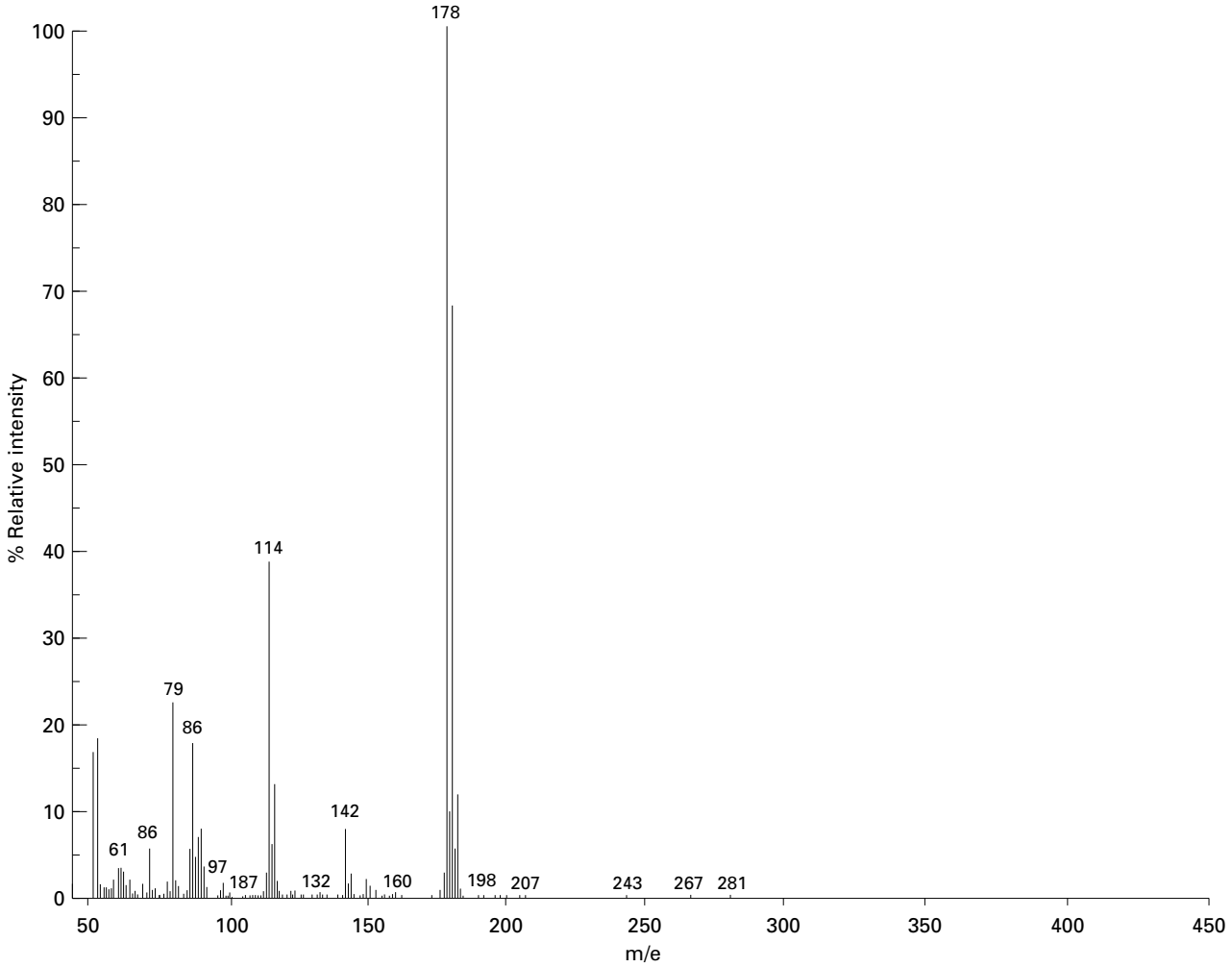

Fig. 4 Mass spectrum of the metabolite isolated from *Alcaligenes eutrophus* TCP cultures

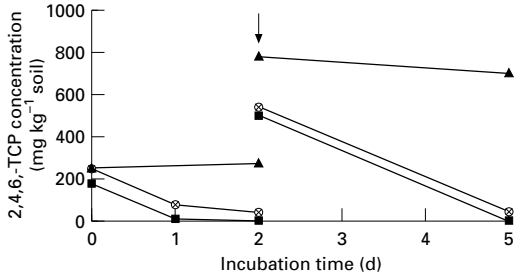

Fig. 5 2,4,6-TCP degradation rate in soil microcosms. ○, soil (S); ▲, autoclaved soil (AS); ■, inoculated soil (IS); ×, autoclaved inoculated soil (AIS). IS and AIS were inoculated with *Alcaligenes eutrophus* TCP at the same cell density (10^8 cfu g^{-1} soil). Arrow indicates 2,4,6-TCP addition (500 mg kg^{-1} soil)

Table 1 Microbial growth in soil microcosms containing 250 mg 2,4,6-TCP kg^{-1} soil and inoculated with *Alcaligenes eutrophus* TCP at different cell densities

Incubation time (Day)	IS	AIS	
	high density	high density	low density
0	$3{\cdot}8 \times 10^8$	$1{\cdot}8 \times 10^8$	$6{\cdot}7 \times 10^6$
2	$1{\cdot}6 \times 10^9$	$1{\cdot}3 \times 10^9$	$1{\cdot}1 \times 10^9$
5	$1{\cdot}0 \times 10^9$	$2{\cdot}6 \times 10^9$	$1{\cdot}9 \times 10^{10}$

IS, Inoculated soil; AIS, autoclaved inoculated soil.
The inoculum was prepared at required cell densities as reported in Materials and Methods. Each count represents the average of two replicates and is expressed as cfu g^{-1} dry soil.

mation in the microbial culture broths of a dichlorinated dioxygenated metabolite, characterized by mass spectrometry, is in accord with the results of other authors with *Pseudomonas* and *Azotobacter* strains (Li *et al*. 1991 ; Kiyohara *et al*. 1992). The removal of the chlorine substituent, mostly in *para* position, by a characterized *p*-hydroxylase to give chlorinated hydroquinones, is retained as the first step in the aerobic degradation of higher chlorinated phenols (from TCPs to PCP) (Tomasi *et al*. 1995).

Laboratory microcosms inoculated with *Alc. eutrophus* TCP cells, performed to check whether the strain could be employed for the decontamination of sites polluted with 2,4,6-TCP, showed that : (a) the degradation of 2,4,6-TCP was a biotic process as it did not occur in the autoclaved soil ; (b) 2,4,6-TCP was removed at the concentration tested from both inoculated and non-inoculated microcosms at almost comparable rates (however, the slightly lower 2,4,6-TCP degradation rate found in both non-inoculated and autoclaved inoculated soil microcosms with regard to the degradation rate found in inoculated soil microcosm, suggested that autochthonous microflora, able to degrade 2,4,6-TCP, might accelerate the degradative process in the inoculated soil microcosm) ; (c) the degradative process was accompanied by bacterial growth in all microcosms tested ; (d) the inoculum sizes used did not influence the rate of the biodegradative process (data not shown), but affected bacterial growth quantitatively (Table 1) (the concentration of 2,4,6-TCP tested could obviously not adequately support the growth of a population with a high initial density) ; (e) no competition between indigenous microflora and *Alc. eutrophus* TCP strain was observed.

In conclusion, the fast removal of 2,4,6-TCP in inoculated microcosms demonstrates that *Alc. eutrophus* TCP can be used successfully in the remediation of soils contaminated with this zenobiotic. However, the degradability of 2,4,6-TCP, biologically mediated, in the soil used in this experimentation, even if not previously treated with chloroorganic pesticides, provides evidence of self-remediability of the contaminated sites, due to the biochemical activities of indigenous micro-organisms. The advantage deriving from this 'self-remediation' is primarily the promptness of the microbial processes which can start as soon as the pollutants are introduced into the environment, as adaptation to the environmental physico-chemical parameters is not required. The assessment of this intrinsic degradative capacity, which can be favoured and accelerated through aeration of the soil, addition of organic nutrients and/or appropriate electron acceptors, allows the adoption of an appropriate remediation strategy. However, the inoculation of contaminated sites with specialized organisms isolated in the laboratory is not always effective as it is strictly dependent on biotic or abiotic stresses (competition with autochtonous micro-organisms, presence of grazing populations, inadequate concentrations of the contaminants etc.).

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