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ORIGINAL PAPER

Isolation and characterization of two new microbial strains capable of degradation of the naturally occurring organophosphonate—ciliatine

Magdalena Klimek-Ochab · Agnieszka Obojska · Anna M. Picco · Barbara Lejczak

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Abstract Air-born mixed fungal and bacterial culture capable of complete degradation of ciliatine was isolated. The utilization of the natural organophosphonate proceeded in the phosphate independent manner. Enzymatic activity involved in ciliatine degradation studied in the fungal cell-free extract proved to be distinct from bacterial pathway described before.

Keywords 2-AEP:pyruvate transaminase · 2-Aminoethylphosphonic acid (2-AEP · ciliatine) · Biodegradation · Carbon–phosphorus bond cleavage · Organophosphonates

Introduction

Microbial capability of degradation of chemical compounds is of increasing use in biological systems for wastes and pollutants removal. Biodegradation studies have been traditionally focused on isolation and characterization of a pure microbial culture. Although this has led to the solving of some metabolic processes involved in mineralization of certain environmental pollutants many xenobiotics are still regarded as resistant to biodegradation (Watanabe and Hammamura 2003). However, it should be borne in mind that biodegradation occurring in an environment exposed to the chemical pollutant is a complex process in which many different metabolically active microbial communities take part. Therefore, the biodegradation studies should be carried out in two directions—first, to bring the experimental conditions closer to the actual in situ parameters by isolating mixed groups of microorganisms and assessing their involvement in the biodegradation process; second, to identify the enzymatic pathways for pollutant degradation in each member of the community (Torsvik and Øvreås 2002). Organophosphonic acids constitute an example of chemical compounds whose environmental fate is of major interest nowadays. Phosphonates, present in the environment either as biogenic or xenobiotic compounds, contain covalent linkage between carbon and phosphorus atom which imposes stability at high temperature, low and high pH and in the presence of oxidants.

Important industrial applications of phosphonates result from their effective chelating activity for di- and trivalent metal ions and crystal growth inhibition. They are also used as popular peroxide bleach stabilizers (Nowack 2003). Apparently due

M. Klimek-Ochab · A. Obojska (✉) · B. Lejczak
Department of Bioorganic Chemistry, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, PL-50370 Wroclaw, Poland
e-mail: agnieszka.obojska@pwr.wroc.pl

A. M. Picco
Department of Land Ecology, Section of Mycology, University of Pavia, I-27100 Pavia, Italy

to the presence of natural phosphonates in the environment microorganisms have evolved the ability to metabolize compounds containing C–P bond mainly as a phosphorus source. In pure culture studies several enzymatic pathways were discovered for the microbial use of phosphonates involving phosphonatase, C–P lyase, phosphonoacetate hydrolase and phosphonopyruvate hydrolase (Kononova and Nesmeyanowa 2001).

This paper describes degradation of a natural phosphonate—ciliatine (2-aminoethylphosphonic acid, 2-AEP) by two air borne bacterial and fungal strains which grew spontaneously on the solid medium containing ciliatine. It is the first report on the ability of a fungal strain to utilize compound containing P–C bond as the only source of nitrogen and phosphorus.

Materials and methods

Chemicals

All chemicals and reagents of highest available purity were purchased from Sigma-Aldrich (Poznan, Poland) unless otherwise stated. DEAE Sephacel was purchased from Amersham Biosciences and desalting gel from BioRad. Culture media components were obtained from POCh (Gliwice, Poland). Gradient grade HPLC solvents were purchased from Merck (Poznan, Poland). Deuterium oxide for NMR analysis was purchased from Dr. Glaser AG (Basel, Switzerland). Aminophosphonic compounds used in the study were synthesized in Chemistry Department of Wroclaw University of Technology, Poland, with the exception of *N*-phosphonomethylglycine, aminomethylphosphonic acid and 2-aminoethylphosphonic acid which were purchased from Sigma-Aldrich (Poznan, Poland).

Media and growth conditions

The strains were cultured in Czapek liquid medium (CDM) containing per liter: glucose 30 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $(NH_4)_2SO_4$ 2.64 g, K_2HPO_4 1 g, HEPES 1 g. Phosphorus and nitrogen sources were omitted when required and replaced with filter-sterilized 2-aminoethylphosphonate at the concentration of 4 mM. Liquid cultures (50 ml in 250-ml Erlenmeyer flasks) were inoculated either with a fungal spore suspension in 0.05% Triton X-100 to a density of 10,000 spores ml^{-1} or bacterial biomass to starting OD650 = 0.05, and incubated at 28°C on a rotary shaker at 100 rpm. To evaluate the fungal growth, fungal mycelia were harvested by vacuum filtration onto Whatman filter paper. Filters were dried in an oven for 48 h at 70°C and dry mass was determined. Bacterial growth rates were estimated by optical density measurements at 650 nm.

Isolation procedure

The strains were originally isolated from contaminated solidified Czapek Dox medium (CDM) containing 4 mM ciliatine as a sole source of nitrogen and phosphorus. Inoculum collected from the plate was used to start liquid CDM culture containing 4 mM ciliatine as a sole source of nitrogen and phosphorus. Culture was incubated on a rotary shaker (100 rpm) at room temperature. Inorganic phosphate concentration changes in the culture broth samples was monitored. When significant phosphate release was detected (1.5 mM within 48 h of growth), culture samples were transferred into the same medium. After three subsequent transfers samples of the culture were spread onto solidified CDM containing 4 mM ciliatine supplied as a sole source of nitrogen and phosphorus.

Characterization of 2-AEP degrading microorganisms

Strains consistently recovered from the actively growing culture were isolated by repetitive streaking onto solidified CDM containing 4 mM 2-AEP supplied as sole nitrogen and phosphorus source. The microorganisms were identified in DSMZ laboratory (Germany). Bacterial isolate was Gram-negative, motile, oxidase-positive, catalase-positive non-sporulating rod. The profile of the cellular fatty acids was typical of the *Alcaligenes*/*Bordetella* group. 16SrDNA partial sequencing revealed a similarity of 99.5% to both subspecies of *Achromobacter xylosoxidans*. The

physiological data pointed to *Achromobacter xylosoxidans* ssp. *denitrificans*.

Morphology characterization of the fungal isolate showed thin-walled, smooth, 3 μm ϕ conidiophores terminated in a verticil of metaluae; phialides up to 10 μm long, cylindric with pointed neck; conidia ellipsoid to subglobose, up to 3 μm long, heavy-walled. The strain was identified as *Penicillium purpurogenum* Stoll.

Analytical methods

2-AEP concentration in the culture medium was determined by HPLC analysis in Beckman Gold Nouveau System apparatus using the tosyl derivatization method of Kawai et al. (1991). ^{31}P NMR analysis of culture supernatants were performed after separating cells by centrifugation and evaporating culture liquid to dryness using IKA RV 06 rotary evaporator. The obtained residue was dissolved in 0.5 ml deuterium oxide and analyzed using DRX Bruker apparatus (Karlsruhe, Germany) operating at 121.50 MHz. Inorganic phosphate was determined by the colorimetric method of Fiske and SubbaRow (1925) according to the manufacturers recommendations using Sigma-Aldrich Inorganic Phosphorus Kit. The protein content was assayed by the method of Bradford (1976) using bovine serum albumin as the standard. All determinations were carried out at least in triplicate.

Preparation of cell-free extracts

Isolates were grown separately in medium containing 4 mM 2-AEP as a sole nitrogen and phosphorus source until early mid-log phase.

Microbial biomass was harvested by centrifugation and washed twice with ice-cold 50 mM Tris–HCl buffer pH 8.0 and disrupted ultrasonically using a Cole Parmer Torbeo 36800 600-W sonicator with six cycles of 30 s sonication and 2 min cooling. Cell debris was removed by centrifugation at 20,000 $\times$ *g* for 15 min at 4 °C. The supernatant was column-desalted by passage through a BioGel P6DG (BioRad) column and loaded onto a DEAE-Sephacel column equilibrated with the same buffer. Retained proteins were eluted with a minimal amount of buffer containing 250 mM NaCl and the resulting extract was again column-desalted.

Cell-free extract activity assays

Carbon-phosphorus bond cleavage activity in cell-free extracts prepared from 2-AEP utilizing microorganisms was assayed in a coupled transamination-dephosphonylation reaction by a modification of the method of Cook et al. (1978). The reaction mixture (1.0 ml final volume) contained (μmol ml^{-1}): Tris–HCl buffer (pH 8.0) 100, $MgCl_2$ 5, pyridoxal–5-phosphate 1, DTT 0.5, EDTA 0.5, 2-AEP 5. Sodium pyruvate, α-ketoglutaric acid, oxalacetic acid were included into the reaction mixture at 10 mM concentration individually or in combinations and tested as possible amino group acceptors. The reaction carried out at 30°C was initiated by the addition of cell-free extract containing 0.5–1 mg of protein.. 200 μl aliquots were collected at time intervals and treated with 20% TCA (50% w/v) and the denatured protein precipitate was removed by centrifugation. The supernatant was immediately assayed for inorganic phosphate.

Products of transamination reaction were identified by thin layer chromatography performed on cellulose coated plates (Merck). Aliquots of enzymatic reactions supernatants were applied and the plate was developed in solvent mixture composed of *n*-butanol: acetic acid : water (12:3:5). The samples were run alongside alanine, aspartate and glutamate standards. Plates were visualized by spraying with 0.2% ninhydrin solution in ethanol and heating at 90°C for 5 min.

Results

Degradation of 2-aminoethylphosphonic acid by mixed culture

Our study aimed to characterize newly isolated microorganisms able to utilize 2-AEP as substrate in a phosphate independent manner. Microbial culture derived from accidentally contaminated medium containing ciliatine allowed of the isolation of two microorganisms, *Achromobacter*

xylosoxidans ssp. *denitrificans*) and *Penicillium purpurogenum* Stoll., which originally appeared as coexisting species. It is not the first time that in our laboratory air-borne P-C degrading microorganisms are found by chance (Bujacz et al. 1995).

The degradative ability towards ciliatine was studies in isolates cultured either separately or concurrently as mixed culture. In each case inoculation of medium containing 2-AEP resulted in cultures effectively removing phosphonic substrate supplied as the only source of nitrogen and phosphorus.

The progress of 2-AEP degradation was traced by measurements of inorganic phosphate liberation into the culture medium and reduction in substrate concentration. HPLC analysis of culture supernatants clearly demonstrated that 2-AEP was removed effectively in all culture variants (Fig. 1). In no case complete removal of 2-AEP was observed and the biodegradative efficiency observed at the ninth day of experiment were 65%, 80% and 90% for fungal, bacterial and mixed culture, respectively. The pattern of 2-AEP removal also differed significantly in the three systems used.

In the case of fungal strain no direct relation between kinetics of 2-AEP removal and rate of phosphate release was observed. ^{31}P NMR analysis of fungal cell-free extract showed the presence of ciliatine (δ = 16.0 ppm) proving the uptake and accumulation of the phosphonic substrate in the fungal cell. Interestingly a significant lag-phase in phosphate release into the culture medium characteristic of isolates grown separately did not occur in mixed culture.

Finally, pH changes of culture broths were followed during microbial growth. Decrease of pH value from 7.2 to around 3 was observed in all the cultures studied (data not shown).

In order to better characterize the ability to degrade carbon-to phosphorus bond by the obtained isolates a series of structurally varied aminophosphonates were tested. None of the compounds supported bacterial nor fungal growth when supplied as sole source of both phosphorus and nitrogen. Fungal strain was able to utilize three out of nine compounds, namely: 3-amino-3-phosphonopropionate, 1-aminoethylphosphonate and 1-amino-1-phenylmethylphosphonate as a sole phosphorus source. Under the same culture

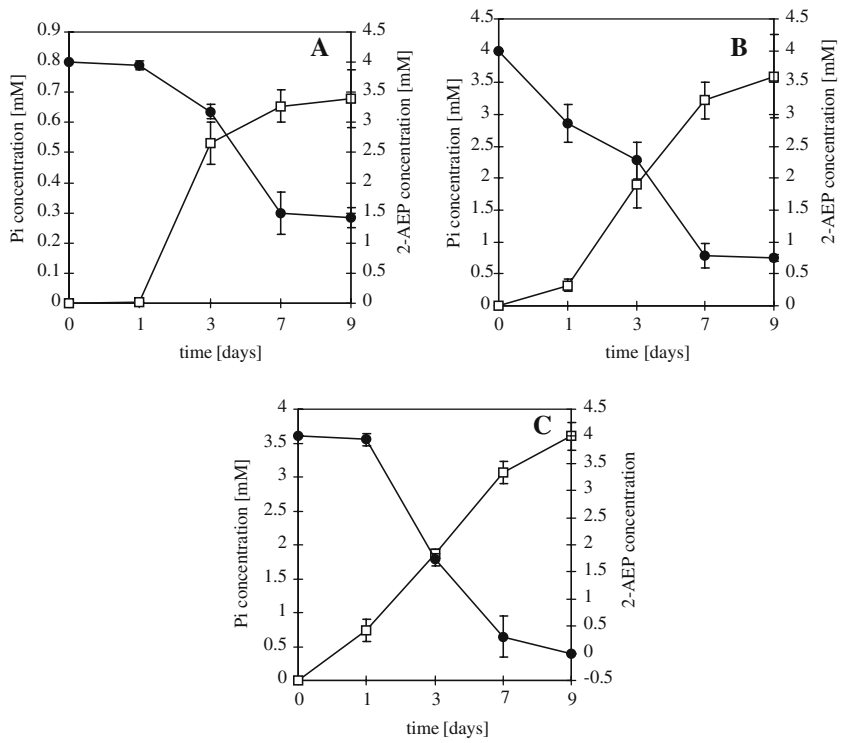


Fig. 1 Utilization of 2-aminoethylphosphonic acid (●) used as a source of phosphorus and nitrogen; release of inorganic phosphate (□) by *P. purpurogenum* (**A**), *A. xylosoxidans* (**B**) and the mixed culture (**C**). Data are means ± SD of three replications

conditions the bacterial isolate showed ability to utilize all compounds except 1-aminomethylphosphonate (Table 1). Degradation of the aminophosphonates utilized by the fungal strain was confirmed by ^{31}NMR analysis. No phosphonic products of degradation carried out by the *P. purpurogenum* Stoll culture were detected after 3 or 4 days of experiment.

Biodegradation of 2-AEP by *Penicillium purpurogenum* Stoll

Penicillium strain was tested for growth on 2-AEP supplied as a sole source of both nitrogen and phosphorus or as nitrogen source in the presence of 2 mM P_i. Fast phosphonate depletion from the culture medium was observed in both cases (Fig. 2). Biomass yields were comparable to those obtained in complete medium containing inorganic phosphate and ammonium ions (data not shown) although, quite surprisingly, only 70% of 2-AEP was degraded when used as a source of phosphorus and nitrogen, whereas complete utilization was observed in the presence of inorganic phosphate.

The effect of rising concentration of 2-AEP on *P. purpurogenum* Stoll growth was studied. Concentration of 2-AEP supplied as nitrogen and phosphorus source up to 15 mM stimulated the fungal growth, however, a strong growth inhibition was observed when the substrate concentration exceeded this value. When increasing concentrations of 2-AEP were supplied as a phosphorus source alone no toxic effect was observed (data not shown).

In the case of *Achromobacter xylosoxidans* ssp. *denitrificans* 2-AEP supported the growth in the concentration ranging from 1 mM to 30 mM regardless of the presence of Pi.

Enzymatic activities of fungal and bacterial cell-free extracts

Cell-free extracts prepared from microbial biomass grown on 2-AEP as the source of nitrogen and phosphorus contained detectable 2-AEP transaminase and phosphonatase activities. Specific activities at substrate concentration of 10 mM ranged from 0.8 nmol to 5.6 nmol of Pi released s^{-1} (mg protein)$^{-1}$ in the case of fungal cell extract and 2–20 nmol of Pi released s^{-1} (mg protein)$^{-1}$ in bacterial protein preparation, depending on amino group acceptor used in reaction mixture. Quite surprisingly the fungal cell extract exhibited the highest activity in the presence of oxalacetic acid and the lowest with sodium pyruvate alone. For bacterial cell-free extract pyruvate was found the preferable amino group acceptor (Table 2) which was in accordance with literature data. The TLC analysis confirmed that aspartate was a product of fungal transaminase activity, whereas bacterial activity resulted in alanine formation.

Discussion

Fungi exhibit enormous potential towards metabolizing a huge variety of natural and xenobiotic compounds to capture energy and nutrients for growth (Adosinda et al. 2001; Bending et al. 2002; Weiland et al. 1995).

Studies concerning metabolism of phosphonates in eukariotic microbial cells are documented by only several reports on the utilization of phosphonates as sole phosphorus source by fungal isolates (Bujacz et al. 1995; Krzyśko-Łupicka et al. 1997; Sobera et al. 1997; Zboińska et al. 1992). The ability of fungal isolates to utilize organophosphonates as sole source of nitrogen are limited to two examples. Bode and Birnbaum described the utilization of the natural compound phosphonoalanine as a nitrogen source by yeast *Candida maltosa* (Bode and Birnbaum 1989), whereas Ternan and McMullan reported the utilization of 4-aminobutylphosphonate as a source of nitrogen by *Kluyveromyces fragilis* (Ternan and McMullan 2000).

This study is the first report on the ability of fungal strain to metabolize a natural aminophosphonate ciliatine as a source of nitrogen and phosphorus in a phosphate independent manner. Ciliatine degradation has always been extensively studied in bacteria and successfully evaluated on the level of regulation, mechanism of catalyzed reactions and enzyme structure. In all reported cases transamination catalyzed by

Table 1 Growth of *Penicillium purpurogenum* and *Achromobacter xylosoxidans* strains in medium containing phosphonic compounds supplied as sole phosphorus source (1 mM)

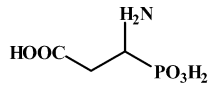
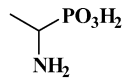
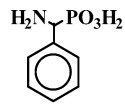
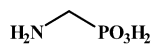
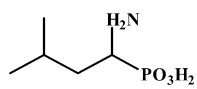
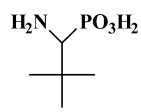
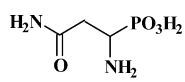
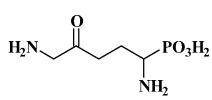
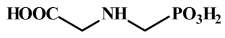
Substrate	*P. purpurogenum* (%)	*A. xylosoxidans* (%)
 3-amino-3-phosphonopropionate	49.2	34
 1-aminoethylphosphonate	60	5
 1-amino-1-phenylmethylphosphonate	96	80
 aminomethylphosphonate	0	53
 1-amino-3-methylbutylphosphonate	0	42
 1-amino-2,2-dimethylpropylphosphonate	0	38
 3-amino-3-phosphonopropionoamide	0	76
 4-amino-4-phosphonobutyramide	0	92

Table 1 continued

Substrate	*P. purpurogenum* (%)	*A. xylosoxidans* (%)
 N-phosphonomethylglycine	0	67

Growth expressed as percent of dry mass for *P. purpurogenum* and optical density for *A. xylosoxidans* compared with values obtained in complete CDM medium

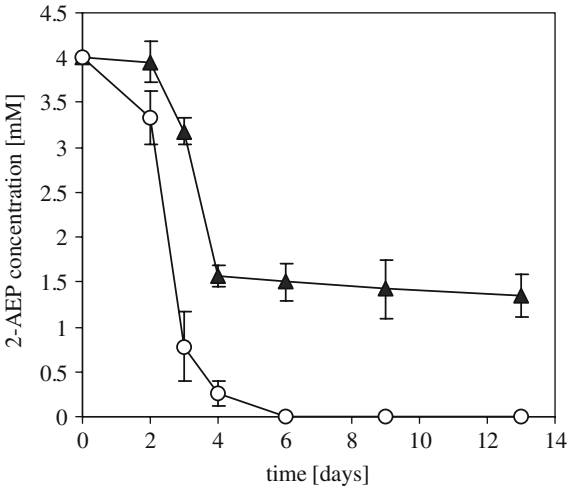


Fig. 2 Comparison of fungal utilization of ciliatine as a sole source of (○) nitrogen in the presence of inorganic phosphate; (▲) nitrogen and phosphorus

Table 2 Specific activity of fungal and bacterial transaminase against 10 mM ciliatine in free cell extracts

The acceptor of amine group	nmol Pi s^{-1} (mg protein)$^{-1}$	
	Achromobacter	Penicillium
Sodium pyruvate	20	0.8
α-ketoglutaric acid	0.9	2.6
Oxalacetic acid	2	5.6

Mean values over at least three replication are reported with SD within 15%

pyridoxal phosphate dependent 2-AEP:pyruvate aminotransferase [EC 2.6.1.37] leads to phosphonoacetaldehyde formation. Phosphonoacetaldehyde hydrolase (phosphonatase) [EC 3.11.1.1] purified from *Bacillus cereus* and *Pseudomonas aeruginosa* catalyses cleavage of the C–P bond due to reaction between active site lysine and carbonyl group in phosphonoacetaldehyde followed by β-elimination process (Olsen et al. 1992).

In this work we describe the newly isolated *Penicillium purpurogenum* Stoll able to utilize 2-AEP as a source of phosphorus and nitrogen regardless of the phosphate status of the cell. Studied *Penicillium* isolate could possess either a Pi-deregulated or a specific phosphonate uptake system, which can transport substrate into the cell in the presence of inorganic phosphate. The degradation of 10 mM 2-AEP in fungal culture was complete only when the aminophosphonate served as a sole nitrogen source and the source of phosphorus was 1 mM phosphate originally supplemented to the medium plus excess phosphate resulting from the C–P bond cleavage released during culture growth (data not shown). When 2-AEP served both as nitrogen and phosphorus source, observed removal of the substrate was reduced (Fig. 2). Quite surprisingly, under these conditions the fungal strain proved to be fragile to ciliatine concentrations exceeding 15 mM. HPLC and ^{31}P NMR analysis of fungal culture supernatant and cell extract performed at the moment when fungal growth on 2-AEP as nitrogen and phosphorus was ceased (4 days of incubation) did not show the presence of any possibly toxic phosphonic by-product of 2-AEP degradation or transformation.

Cell-free extracts of fungal strain possessed enzymatic activity for 2-AEP degradation. The pathway of 2-AEP degradation in *Penicillium* isolate seemed to involve transaminase activity (activities) different from bacterial enzyme described in literature (Wanner 1996). Bacterial enzyme from various sources was specific for pyruvate, whereas in *P. oxalicum* cell-free extract pyruvate, α-ketoglutarate and oxalacetate served

as acceptors of amino moiety, with the latter yielding the highest activity (Table 2).

The ability of *Penicillium* isolate to utilize structurally diverse aminophosphonates was studied. All the compounds which supported the fungal growth as the sole phosphorus source were phosphonic analogues of α-amino acids, thus the mode of their degradation was certainly different from that found for 2-AEP (Table 1).

The second isolate studied in this work *Achromobacter xylosoxidans* grew spontaneously along with *Penicillium purpurogenum* on solid medium containing ciliatine and was able to degrade this aminophosphonate effectively by the known degradative pathway (transamination of 2-AEP followed by C–P bond cleavage of the formed phosphonoacetaldehyde). Evident preference towards pyruvate was noticed in the bacterial cell extract transaminase activity. The activity of the bacterial enzyme was much higher than of the fungal protein preparation. *A. xylosoxidans* was also less substrate specific if considering its ability to cleave C–P bonds in other aminophosphonate substrates. The growth was supported exceptionally by nearly all the studied aminophosphonates and only in the case of phosphonic acid analogue of alanine minute growth was observed. Oppositely to fungal isolate it was able also to cleave this bond in two environmentally important compounds—herbicide glyphosate (*N*-phosphonomethylglycine) and its metabolite (aminomethylphosphonic acid). Such a wide substrate specificity seems to indicate that this isolate possesses more than one metabolic pathway of C–P bond cleavage.

Our study also clearly indicated some differences in phosphonate degradation carried out by isolates separately and in mixed culture. Airborne *Penicillium purpurogenum* strain and *Achromobacter xylosoxidans* grew spontaneously on solid medium containing 2-AEP. Therefore, it is not surprising that most intensive phosphonate depletion from the culture medium took place in the culture containing both isolates. Because the substrate degradation was most quickly initiated by bacterial strain (Fig. 1) it may suggest its involvement in the initial step of the substrate decomposition thus promoting the growth of fungus by providing inorganic phosphate. The kinetics of xenobiotics decomposition carried out by co-operating mixed microbial population differs significantly from processes which depend upon pure culture catabolic potential. Thus this clearly indicates that the fate of xenobiotics in natural environment may not be reflected by those observed in laboratory studies.

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