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**BIODEGRADABILITY OF TETRACHLOROBENZYL TOLUENES AND
POLYCHLORINATED BIPHENYLS IN ACTIVATED
SLUDGE AND IN CULTURES OF *ALCALIGENES* SP. JB1:
A PRELIMINARY STUDY**

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ABSTRACT

The biodegradability of tetrachlorobenzyltoluenes (TCBTs) and polychlorinated biphenyls (PCBs) was investigated in activated sludge and in batch cultures of the PCB-degrading microorganism *Alcaligenes* sp. JB1. There was no evidence for significant biodegradation of either TCBTs or PCBs in activated sludge. In contrast, cultures of strain JB1 did degrade PCB 40, but again there was no evidence for degradation of TCBTs. Although no final conclusions can be drawn from these results about the biodegradability of TCBTs, they do indicate that TCBTs are environmentally persistent compounds.

INTRODUCTION

In the current study an effort is made to investigate the biodegradability of TCBTs. TCBTs, commercially known as Ugilec 141, are used as PCB replacements in the mining industry in Germany. Theoretically, 96 different tetrachlorobenzyltoluenes are possible (Fig.1).

Figure 1. Molecular structure of TCBTs.

TCBTs released underground can enter the environment via pit water, mine outputs or ventilation systems and as a consequence they can contaminate the food chain, especially fish. Significant levels of TCBTs are found

in the rivers Lippe and Ruhr, which are located near areas with extensive underground mining (1-5). The presence of TCBT residues in the environment suggests that TCBTs as a class of compounds are resistant to microbial degradation. Few data, however, are reported on the biodegradability of TCBTs. Bakes et al. (6) found a biodegradability of 60-70% after 180 days for Ugilec 141, which consists of 69 TCBT isomers, and a biodegradability close to zero for penta- and hexachlorobiphenyls under the same conditions.

Several methods have been defined by the EC (7) and the OECD (8) for the initial screening of the ready biodegradability of new chemicals. However, no single ready biodegradability test can be the most suitable for all chemicals. Ready biodegradability tests are very stringent and provide limited opportunity for biodegradation and acclimatization to occur for recalcitrant and poorly-soluble chemicals as TCBTs and some PCBs. Therefore, it is justified to perform other tests to demonstrate the biodegradability of a chemical. Simulation tests are designed to investigate the biodegradability of the test compounds under environmentally relevant conditions. Inherent biodegradation tests under favourable conditions can be used to assess whether a chemical has any potential for biodegradation (8).

Several studies reported PCBs to be degradable by activated sludge microorganisms, decreasing with increasing number of chlorine substituents (9,10). In this study the susceptibility of TCBTs to primary degradation by activated sludge microorganisms is compared with reported primary degradation of PCBs. Two tests with activated sludge are performed to give an indication about the biodegradability of TCBTs in the environment compared to that of the structurally related PCBs. TCBTs and 4'-mono-, 4,4'-di- and 2,2',4,4',6,6'-hexachlorobiphenyl are used as test compounds.

Secondly, an inherent batch culture biodegradability test was carried out using optimum conditions to assess whether TCBTs have any potential for biodegradation. The primary importance of the inherent biodegradability of persistent polychlorinated biphenyls (PCBs) has led to many studies regarding microbial degradation of PCBs (e.g. 11-15). Although PCBs do not occur naturally in the environment, several bacteria have been isolated which can degrade PCBs. These bacteria are often unable to grow on halogenated aromatic compounds, but are able to degrade them while growing on other organic compounds (cometabolism). Bacteria able to degrade PCBs aerobically are from the genera *Pseudomonas*, *Alcaligenes*, *Arthrobacter* and *Acinetobacter*. In all PCB-degrading strains the major pathway by which PCBs are biodegraded involves initial dioxygenation to form metabolites hydroxylated in the 2 and 3 positions which are degraded in turn by *meta* ring cleavage to ultimately give chlorinated benzoic acids (11-17). In previous reports *Alcaligenes* strains were capable of cometabolizing a variety of PCB compounds (13,14,17-19). Furukawa et al. (18) reported that tetrachlorobiphenyls with two chlorines substituted on each ring showed very poor degradability. 2,2',3,3'-Tetrachlorobiphenyl, however, was degraded rapidly by *Alcaligenes* Y42 (18). *Alcaligenes* sp. JB1 is able to cometabolize PCBs containing up to six chlorine substituents (17,19). Batch cultures of *Alcaligenes* sp. JB1 were used in two experiments to investigate the comparative inherent biodegradability of eight TCBT isomers and 2,2',3,3'-tetrachlorobiphenyl, using cultures grown on either biphenyl or diphenylmethane as carbon sources.

MATERIALS AND METHODS

Microorganisms and cultivation

The activated sludge was obtained from a local municipal sewage treatment plant. The medium used in the activated sludge experiments was the same as the medium used for the batch cultures of strain JB1.

Strain JB1 was isolated from soil by selective enrichment using biphenyl as carbon source. The organism was identified as an *Alcaligenes* strain by means of the API-20NE test system. Cultivation was carried out in the following mineral medium supplemented with biphenyl or diphenylmethane as the sole carbon source: $\text{KH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (30 mM), NH_4Cl (50 mM), $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ (1.0 mM), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.625 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01 mM), $\text{N}(\text{CH}_3\text{COOH})_3$ (1 mM) and a trace elements solution (Consisting of ZnO (12.7 μM), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (50 μM), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 25.3 μM), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (2.54 μM), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (5 μM), HBO_3 (2.58 μM),

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.046 μM)). The final pH of the medium was 7.2. The organisms were grown for 3 or 4 days, and the culture fluid was filtered once to remove remaining solid biphenyl or diphenylmethane.

Chemicals

Nine TCBTs were purchased from Promochem (Wesel, Germany); no 28, (numbers according to Ehmann and Ballschmiter (20)), 2,2',4,6'-5; no 25, 2,2',4,5'-5 ; no 36, 2,2',5,5'-4 ; no 22, 2,2',4,4'-5; no 27, 2,2',4,6'-3; no 80, 2',3,4,6'-6; no 21, 2,2',4,4'-3; no 52, 2,3',4,4'-5; no 74, 2',3,4,4-6. PCBs were obtained from Promochem; no 3 (IUPAC-numbering) 4'-PCB; no 15, 4,4'-PCB; no 40 2,2',3,3'-PCB; no 101, 2,2',4,5,5'-PCB; no 155 2,2',4,4',6,6'-PCB. Silica (60, 70-230 mesh) was purchased from Merck. n-Hexane, 2,2,4-trimethylpentane and acetone were obtained from Janssen Chimica, Rathburn and Merck respectively. All solvents were redistilled before use.

Experimental set-up

Activated sludge In the first experiment 1 mL activated sludge and 1 mL solution of 8 TCBTs in acetone were added to each of three 250 ml flask containing 100 mL medium. The nominal concentration of the TCBTs were in between 1 and 4 $\mu\text{g L}^{-1}$. To enable detection of any abiotic loss of material, three control flasks were prepared in the same way, without adding sewage sludge. Sewage sludge and medium were checked for the amount of TCBTs in three blank flasks. All flasks were incubated in the dark at 20°C on a rotary shaker at 100 rpm.

In the following experiment the comparative primary degradation was investigated for both PCBs and TCBTs in activated sludge. 4 PCB-congeners and 8 TCBT-isomers were added together to 1 litre activated sludge. The nominal concentrations of PCB 3, 15 and 155 were 110, 8.4 and 0.6 mg L^{-1} respectively. A control flask was prepared in the same way in the absence of bacteria by acidification with concentrated H_2SO_4 .

Batch cultures Two separate experiments were done with *Alcaligenes* sp. JB1. In the first experiment the strain was grown on biphenyl as carbon source and TCBT 52 and PCB 40 as test chemicals. The nominal concentrations of TCBT 52 and PCB 40 were 2 and 3 mg L⁻¹ respectively. A second experiment was performed under identical conditions with the exception of the carbon source and the number of TCBTs as test chemicals. In this case diphenylmethane was used as a carbon source and 8 TCBTs were used in the second experiment as well as PCB 40 (nominal concentrations in between 150 and 400 µg L⁻¹). The ninth TCBT isomer (no 36) was used as an internal standard.

For both experiments the cometabolism of TCBTs and PCB 40 was studied in 250 ml Erlenmeyer flasks with stoppers, which were porous to air. Per experiment three flasks were prepared for 1 or 8 TCBTs respectively, three flasks for PCB 40 and three flasks for control experiments. The experiment was started once bacterial growth was visible. TCBTs and PCBs were added as acetone solutions to the flasks, containing 100 mL medium, 0.1 g carbon source and 1 mL inoculum of a culture of JB1. Control experiments were carried out under identical conditions in the absence of bacteria by acidification with concentrated H₂SO₄.

Sampling, extraction and clean-up

In the first experiment with activated sludge whole samples were taken after 28 days of incubation. In the second experiment with activated sludge triplicate 50 ml samples were taken after 0, 1, 2 and 4 weeks of incubation. Duplicate sampling (10 mL) from the batch cultures of JB1 took place after 0, 6 and 70 hours under magnetic stirring. The samples were extracted with 100, 50 and 10 ml n-hexane respectively, after addition of TCBT 36 as an internal standard and acidification with H₂SO₄. The hexane layer was removed after centrifugation (30 min., 2000 rpm) and gently reduced in volume under a nitrogen stream to approximately 1 mL or diluted to 10 mL depending on the sample. The extract was then passed through a silica column for the batch culture samples or through two columns, a silica and an aluminium oxide column, for the activated sludge samples. The silica column consisted of 0.7 g activated silica and 15 mL n-hexane was used for elution. The organic fraction on the aluminium oxide column (Na₂SO₄, 1 g Al₂O₃, Na₂SO₄, 2 g silica) was eluted with 35 mL n-hexane. The samples were concentrated to approximately 1 mL. After adding PCB 155 or PCB 101 as injection standard, the eluate was ready for analysis.

The internal standard TCBT 36 was used to correct for losses during extraction and clean-up. In all samples processed TCBT was invariably recovered in an amount of greater than 50%. To obtain an indication of losses during separate steps of the analytical procedure recoveries of a hexane solution spiked with a mixture of TCBTs were determined. A mean recovery value of 68% and 86 % were found for the extraction and silica column clean-up, respectively.

Analysis

For analysis, 1 µL of the extract was injected automatically into a Hewlett-Packard 5890A gas chromatograph, equipped with a ⁶³Ni electron capture detector and an on-column injector with a Carlo Erba A200S autosampler. The GC was connected to a HP 3396A integrator. A 30-m J&W DB-5, 0.32 mm internal diameter, fused silica capillary column (film thickness, 0.25 µm) was used with a 2 m 0.53 mm retention gap. The carrier gas

was helium and the make-up gas was Ar/CH₄ 0.5-5%. The temperature program was as follows: 120°C - 0.4 min. - 70°C/min. - 200°C - 1 min - 1°C/min. - 211°C - 0 min - 40°C/min - 320°C - 6 min.

RESULTS AND DISCUSSION

The results of the first experiment with activated sludge are shown in Fig. 2. The concentrations are averages of three samples with standard deviations. As no differences in concentration can be seen between control and culture flasks, this may indicate that the TCBTs were not degraded.

Figure 2. Primary degradation of TCBT isomers by activated sludge after 28 days of incubation.

In the second experiment the biodegradability of TCBTs in activated sludge is compared with the biodegradability of PCBs, whose biodegradability in activated sludge is reported in the literature. Several studies (9,10) reported the capability of activated sludge to degrade mono- and dichlorobiphenyl isomers or mixtures. Higher chlorinated biphenyls were found to be more resistant. The results of the comparative degradation of six TCBTs and mono-, di-, and hexachlorobiphenyls in activated sludge are shown in Fig. 3A, B, and C. The concentrations are averages of three samples. No degradation of TCBT isomers nor PCB isomers was shown in activated sludge during the four weeks of incubation. This activated sludge did not seem to be able to biodegrade mono- and dichlorobiphenyls. The activated sludge, which was previously reported to be able to biodegrade mono- and dichlorobiphenyls (9,10), may have been exposed to these PCBs for a long time. Obviously, the biodegradation of TCBTs and PCBs is not a general occurring phenomenon. It is possible that these compounds are only biodegraded in activated sludge after long term exposure and adaptation.

A concentrations of PCBs (no 3, 15) in culture and control flasks

B concentrations of TCBT isomers in culture flasks

C concentrations of TCBT isomers in control flasks

Figure 3. Primary degradation of PCBs and TCBTs by activated sludge:

For compounds such as PCBs and TCBTs it may be preferable to perform an inherent biodegradability test instead of a ready biodegradability test, and thus to create favourable circumstances and the availability of the right microorganisms. In this study the microorganisms used in the inherent biodegradability tests are *Alcaligenes* sp. JB1, which is capable of degrading compounds with comparable structures to TCBTs, namely PCBs (13,14,17-19). The data shown in Fig. 4 are results of the first batch experiment using biphenyl as carbon source and TCBT 52 and PCB 40 as test compounds. Concentrations are the mean of two samples. PCB 40 seems to be degraded in culture flasks, as it almost disappeared after 72 hours of incubation. However, some decline can also be noticed in control flasks, which may be caused by physical chemical processes. Compared with PCB 40 no obvious decline in concentration of TCBTs is shown in Fig. 4 for both culture and control flasks. This may be an indication that PCB 40 is more susceptible than TCBT 52 to biodegradation by *Alcaligenes* sp. JB1.

Figure 4. Degradation of TCBT 52 and PCB 40 by *Alcaligenes* sp. JB1, grown on biphenyl.

Fig. 5 A and B show the results of the second batch experiment. The concentrations are the mean of two samples. In this experiment the bacteria were grown on diphenylmethane, a compound whose structure is more similar to that of TCBTs and therefore may influence the kind of enzymes that are induced. 8 TCBTs and PCB 40 were used as test compounds. Comparing the concentrations in the culture and control flasks for both TCBTs and PCB 40, reveals that PCB 40 is almost completely degraded during the incubation time. Again no obvious decline in concentrations can be seen for TCBTs in the culture flasks. This may be a further indication that TCBTs are less biodegradable than PCB 40 or that the conditions in the batch culture seem to be more favourable for the degradation of PCB 40 than for the degradation of TCBTs. The biodegradation of PCB 40 by *Alcaligenes* sp. JB1 is in agreement with literature reports (19).

Figure 5. Degradation of 7 TCBTs and PCB 40 by *Alcaligenes* sp. JB1, grown on diphenyl methane

In all experiments the initial concentrations of the test compounds determined were lower than the nominal concentrations of these compounds added to the experiments. These losses may be caused by physical chemical phenomena, e.g. sorption processes.

CONCLUSIONS

Even under favourable circumstances, such as in cultures of *Alcaligenes* sp. JB1, capable of degrading the structurally related PCBs, grown on diphenylmethane, TCBTs were not biodegraded in this study. This is in disagreement with the reported degradation of Ugilec 141 (6), but in accordance with the persistence and

bioaccumulation of TCBTs in the environment (1-5). The experiments with batch cultures of *Alcaligenes* sp. JB1 show a slight indication that PCB 40 is more susceptible to biodegradation than TCBTs. Both TCBTs and PCBs, even mono- and dichlorobiphenyls, seem to be persistent in the activated sludge used in this study. Based on these results, we assume that the biodegradation rate of TCBTs is zero. This assumption will be used in environmental distribution models as input data about the biodegradability of TCBTs. Evidently, more research is needed on the biodegradation of TCBTs as these experiments are only preliminary. Besides aerobic biodegradation, the possibility of anaerobic dechlorination of TCBTs should be involved in future research.

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