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EXPERIMENTAL ARTICLES

Degradation of *ortho*-Chlorophenol, *para*-Chlorophenol, and 2,4-Dichlorophenoxyacetic Acid by the Bacterial Community of Anaerobic Sludge

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Abstract—The bacterial community of anaerobic sludge could degrade *o*-chlorophenol, *p*-chlorophenol, and 2,4-dichlorophenoxyacetic acid at concentrations as high as 100 mg/l. The time needed for the degradation of a given chlorinated phenol derivative increased 1.5- to 2-fold upon a twofold increase in its concentration (from 50 to 100 mg/l). The duration of the adaptation period depended on the compound studied and on its concentration. The degradation of 2,4-dichlorophenoxyacetic acid proceeded via 2,4-dichlorophenol and *p*-chlorophenol as intermediates; the degradation of *o*-chlorophenol occurred with the formation of phenol. The dynamics of *p*-chlorophenol degradation and chloride ion accumulation were studied.

Key words: degradation, bacterial community, anaerobic sludge, chlorinated phenol derivatives.

The problem of environment decontamination from stable xenobiotics, including halogenated organic compounds widely used in industry and agriculture, is currently urgent. Therefore, much attention is paid to research into the biochemistry of bacterial degradation of various halogenated xenobiotics, both aerobic [1, 2] and anaerobic [3, 4]. Chlorinated phenol derivatives are widely used in agriculture as pesticides and belong to the most stable xenobiotics. By now, sufficient data have been accumulated on the degradation of mono-, di-, and trichlorophenols by aerobic bacteria [6, 7], but only a few works have been devoted to the bacterial degradation of phenol and its chlorinated derivatives under anaerobic conditions [8, 9]. The latter processes deserve attention, since one of the most widely used pesticides is 2,4-dichlorophenoxyacetic acid (2,4-D), whose degradation results in the accumulation of 2,4-dichlorophenol, *p*-chlorophenol (*p*-CP), and *o*-chlorophenol (*o*-CP).

MATERIALS AND METHODS

This work used activated sludge samples taken from sludge pits of chemical plants in Saratov and sludge samples from agricultural irrigation systems (a total of ten samples). Sludge samples were put into 3-l bottles and delivered to the laboratory.

To obtain enrichment cultures, the method of anaerobic cultivation under nitrogen [10] was used. To eliminate traces of oxygen, nitrogen was passed through a column with granulated copper heated to 300°C. Sludge samples were introduced (10 vol %) into 500-ml air-tight flasks, which were then sparged with nitrogen for 5 min; after that, 150 ml of mineral medium was introduced, which contained (g/l) $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.16; $K_2HPO_4 \cdot 3H_2O$, 0.42; $Na_2HPO_4 \cdot 2H_2O$, 2.2; $NaH_2PO_4 \cdot H_2O$, 1.2; $MgSO_4 \cdot 7H_2O$, 0.1; $NaHCO_3$, 2.0; and $Na_2S \cdot 9H_2O$, 0.5. The flasks were sealed hermetically.

The enrichments were incubated for 3–5 days at 30°C. After the appearance of methane in the gas phase, which was indicative of the establishment of anaerobic conditions, the enrichments were supplemented with the compounds studied at concentrations of 50, 100, and 500 mg/l. Variants containing twice autoclaved (121°C, 30 min) sludge samples served as controls.

Methane was determined on an LKhM-80 gas chromatograph equipped with a 0.75 × 3 mm column with Porapak QS (room temperature, argon as a carrier gas).

The compounds under study and their metabolites were determined spectrophotometrically (Specord M-40, Germany), by gas chromatography, and by thin-layer chromatography (Silufol UV-254 plates, Czech Republic). For phenol determination, a benzene–methanol–acetic acid (45 : 8 : 4) mixture was used as the solvent system and the Gibbs reagent and diazotized sulfanilic acid were used as developing agents [11]. Thin-layer chromatography of 2,4-dichlorophenol was performed using *n*-butanol saturated with 5 M NH_4OH as the solvent and diazotized sulfanilic acid as the developing agent [11, 12]. Thin-layer chromatography of *p*-CP was carried out using a benzene–acetone–acetic

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Table 1. Adaptation of the bacterial community of anaerobic sludge to chlorinated phenols

Compound	2,4-Dichlorophenoxyacetic acid			*o*-chlorophenol			*p*-chlorophenol		
Concentration, mg/ml	50	100	500	50	100	500	50	100	500
Adaptation period, weeks	22–24	24–30	45	3–4	3–5	45	8–10	9–10	45
Degradation period, weeks	10–12	20	–	2–3	3–6	–	5–7	10–11	–

Note. "–" means that degradation did not occur.

Table 2. Degradation product of 2,4-dichlorophenoxyacetic acid

Compound	R_f in butanol saturated with 5 M NH_4OH	Spot staining by diazotized sulfanilic acid and 10% K_2CO_3	UV absorption maximum, nm
Degradation product	0.74	Yellow	285.2
2,4-Dichlorophenol	0.74	Yellow	285.0

Table 3. Dechlorination product of 2,4-dichlorophenol

Compound	R_f in benzene–acetone–acetic acid (90 : 10 : 2)	Spot staining by		Retention time in gas chromatography
		5% $AgNO_3$	Gibbs reagent	
Degradation product	0.7	Black	Blue	5′10″
p-Chlorophenol	0.7	Black	Blue	5′10″

Table 4. Degradation product of *o*-chlorophenol

Compound	R_f in benzene–methanol–acetic acid (45 : 8 : 4)	Spot staining by		UV absorption maximum, nm
		Gibbs reagent and 10% K_2CO_3	iazotized sulfanilic acid	
Degradation product	0.62	Blue	Yellow	273.2
Phenol	0.62	Blue	Yellow	273.2

acid (90 : 10 : 2) mixture as the solvent system and the Gibbs reagent and $AgNO_3$ as developing agents [13].

2,4-D and *p*-CP were determined by gas chromatography as methylated derivatives. Samples were methylated with diazomethane in methanol for 20 min at room temperature. 2,4-D was determined on a Chrom 5 chromatograph, using Inerton AW-DMCS (0.200–0.250 mm) with the XE-60 (5%) liquid phase; the temperatures of the column, injector, and detector were 180, 240, and 250°C, respectively; helium was the carrier gas.

p-CP was determined on a Biochrom 1 chromatograph equipped with quartz capillary columns with PEG-20M and a flame ionization detector; the temperatures of the column, injector, and detector were 170, 220, and 230°C, respectively; helium was the carrier gas (1.3 ml/min); P_{inj} was 1.7 atm; the flow distribution was 1 : 40; $CHCl_3$ was the solvent; the sample volume was 1 μl.

The concentration of chloride ions released during the decomposition of chlorophenols was determined photometrically with $AgNO_3$ [14] and potentiometrically using a chlorine-selective electrode [15].

RESULTS AND DISCUSSION

In the mineral medium inoculated with activated sludge, the decomposition of 2,4-D, *o*-CP, and *p*-CP occurred. Degradation of the chlorinated derivatives of phenol occurred in all variants, regardless of the sampling source. In experimental variants with concentrations of 50 and 100 mg/l, these compounds eventually completely disappeared. Degradation of *o*-CP took 2 to 3 weeks at a concentration of 50 mg/l and 3 to 6 weeks at 100 mg/l. *p*-CP was completely decomposed in 5 to 7 weeks at a concentration of 50 mg/l and in 10 to 11 weeks at 100 mg/l (Table 1). 2,4-D was decomposed most slowly, in 10 to 12 weeks at a concentration of 50 mg/l and in 20 weeks at 100 mg/l.

The appearance of the first products of xenobiotic degradation is known to be preceded by a lag period [16, 17], which can be as long as six months and even longer [18]. The duration of the adaptation period was long for all of the chlorinated derivatives we studied and was concentration-dependent (Table 1). The duration of the adaptation period changed insignificantly when the concentrations of the chlorinated phenol derivatives were varied within the range of 50–100 mg/l. For 50 mg/l 2,4-D, the adaptation period lasted 22 to 24 weeks and was two times longer than the period of complete degradation of this compound. For 100 mg/l 2,4-D, the duration of the adaptation period was 24 to 30 weeks (4 to 6 weeks longer than the period of complete degradation). If the 2,4-D concentration was increased to 500 mg/l, no degradation occurred within the entire period of observation (45 weeks). The adaptation to *p*-CP and *o*-CP occurred much faster than the adaptation to 2,4-D. For *o*-CP, the adaptation period was approximately as long as the period of complete degradation; it lasted 3 to 4 weeks at a concentration of 50 mg/l and 3 to 5 weeks at 100 mg/l. For *p*-CP, the duration of the adaptation period was 8 to 10 weeks, both at 50 and 100 mg/l. At a *o*-CP or *p*-CP concentration of 500 mg/l, no degradation occurred.

Degradation of 2,4-D was accompanied by a shift in the absorption maximum of the incubated samples from 283.4 nm (a peak typical of 2,4-D) to 285.3 nm (Fig. 1). A compound was detected whose R_f value in the solvent system used coincided with the R_f of 2,4-dichlorophenol (Table 2); no free chloride ions were found in the medium. The appearance of *p*-CP in the medium was accompanied by the appearance of chloride ions (Table 3). Further conversion was associated with the disappearance of compounds having an absorption maximum within the 200–300 nm range (Fig. 1) and with the further release of chloride ions. By the end of incubation, the amount of chloride ions in the variant with 100 mg/l 2,4-D was 32 mg/l, which corresponded to the complete dechlorination of 2,4-D. We failed to identify products of the further destruction of 2,4-D, most probably because of their rapid decomposition.

The degradation of *o*-CP was accompanied by the release of chloride ions in the medium (28 mg/l at an initial *o*-CP concentration of 100 mg/l) and the appearance of a compound identified as phenol (Table 4). The degradation of *p*-CP was accompanied by the release of a stoichiometric amount of chloride ions (Fig. 2); however, phenol was not detected in this case.

Thus, microbial communities of sludges are capable of the anaerobic degradation of phenol derivatives. The first stages of 2,4-D degradation occur via acetate cleavage resulting in the formation of 2,4-dichlorophenol, which is then dechlorinated. Degradation of *o*-CP begins with the splitting of chloride ions without cleavage of the aromatic ring; dechlorination of *p*-CP is most probably accompanied by aromatic ring cleavage. We

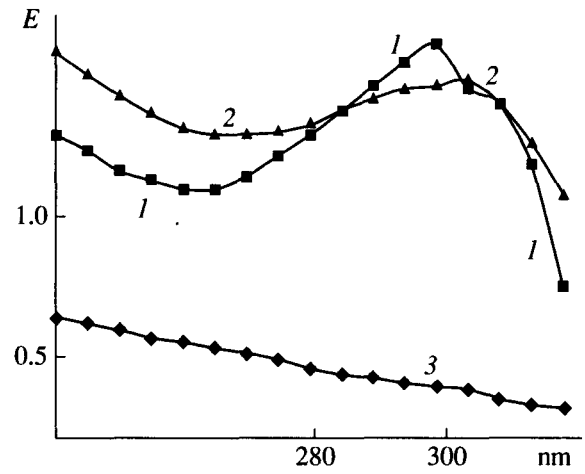

Fig. 1. Spectral changes during the degradation of 2,4-dichlorophenoxyacetic acid: (*1*) the spectrum prior to incubation; (*2*) the spectrum in the course of incubation; (*3*) the spectrum after incubation.

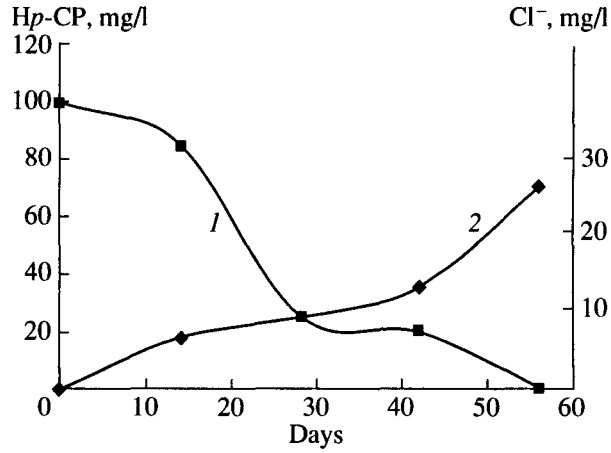

Fig. 2. Degradation of *p*-chlorophenol (*p*-CP) by the bacterial community of anaerobic sludge: (*1*) *p*-CP; (2) chloride ions.

are as yet unable to describe the mechanisms of the anaerobic degradation of chlorinated phenol derivatives exactly. It is noteworthy that the communities of sludges of various origin, including those that had not been in contact with the xenobiotics studied, were able to adapt to the anaerobic degradation of these compounds. There are various ideas about the processes occurring during the adaptation period. Schmidt *et al.* [19] emphasize the possibility of genetic mutations in microbial cells, Stephenson *et al.* [20] consider the synthesis of degradative enzymes to be the most important, and Lewis *et al.* [21] claim that xenobiotics can be used by microbial communities only after the exhaustion of readily available sources of carbon and energy.

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