

# Anaerobic Toxicity and Biodegradability of Hydrolysis Products of Chemical Warfare Agents

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Received January 13, 1999; Revised May 11, 1999; Accepted May 11, 1999

## Abstract

The toxicity and biodegradability of the main hydrolysis products of chemical warfare agents were investigated under methanogenic conditions. Among the tested substances, only MPhA does not have any toxic effect with regard to the acetoclastic methanogenic activity. The toxicity of other compounds varied between moderate (TDG, mercaptoethanol) to strong (ethanolamine, diisobutyl ester of MPhA). Biodegradability tests showed that all the products of chemical detoxification of mustard gas (ethanolamine, ethylene glycol, TDG, mercaptoethanol) can be biomineralized under methanogenic conditions. On the contrary, phosphorus-containing compounds from the chemical detoxification of nerve warfare agents (Sarin, Soman, V<sub>x</sub>-gases) are quite persistent under these conditions.

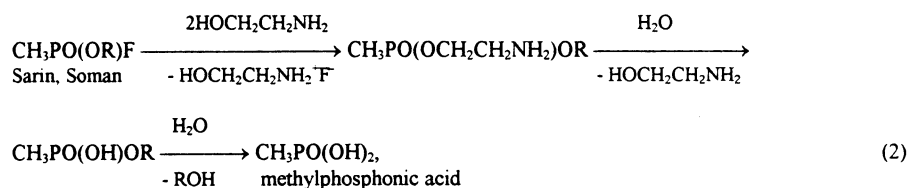
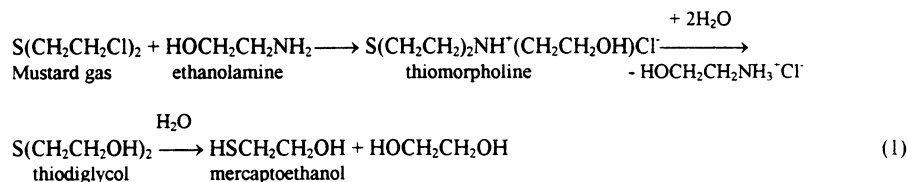
**Index Entries:** Anaerobic biodegradation; chemical warfare; ethanolamine; mercaptoethanol; methylphosphonic acid; thiodiglycol; toxicity.

## Introduction

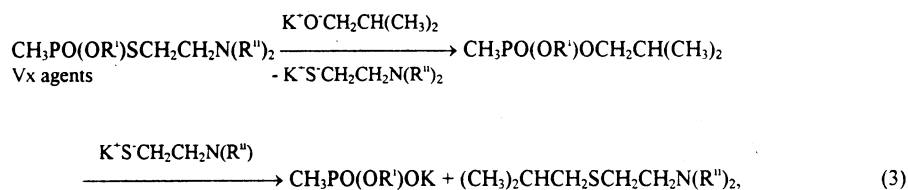
Two types of chemical substances are predominant among the chemical warfare agents: organic compounds with either C-S (mustard gas) or C-P (Sarin, Soman, V<sub>x</sub>-gases) bonds (1,2). To date, several strategies have been proposed for elimination of chemical warfare (3–5). Incineration is the finally accepted way by authorities in the United States, whereas Russia intends to use a chemical hydrolysis (detoxification) of warfare agents

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followed by bituminization of the less toxic hydrolysis products and land-filling in special areas (4). As an alternative to these approaches, the more ecologically sound biocatalytic degradation of original warfare agents or their hydrolysis products to simple organic and inorganic substances has also been proposed (3–5). In the latter case, chemical detoxification can be used as a pretreatment step. The typical chemical reactions that are proposed for use in Russia for this step are the following:



where R = -CH(CH<sub>3</sub>)<sub>2</sub>, -CH(CH<sub>3</sub>)C(CH<sub>3</sub>)<sub>3</sub>



where R<sup>1</sup> = -CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>, R<sup>2</sup> = -C<sub>2</sub>H<sub>5</sub> (Russian V<sub>x</sub>); R<sup>1</sup> = -C<sub>2</sub>H<sub>5</sub>, R<sup>2</sup> = -CH(CH<sub>3</sub>)<sub>2</sub> (USA V<sub>x</sub>).

As can be seen from Eqs. 1–3, the main products of the detoxification are thiodiglycol (TDG), mercaptoethanol, ethanolamine, ethylene glycol (mustard gas detoxification), methylphosphonic acid (MPHA) and its esters, as well as some linear or branched aliphatic alcohols (Sarin, Soman, and V<sub>x</sub> agent detoxification). Not so many microorganisms are known to degrade these substances with C-S or C-P bonds. Among the TDG-degrading microorganisms, the most studied is *Alcaligenes xylosoxidans*, a Gram-negative obligate aerobic bacterium (6,7). The optimal growth conditions for *A. xylosoxidans* using TDG as a sole carbon source are 30°C and pH 8.0. This microorganism exhibits substrate-inhibited growth in batch cultures with a notable decrease in cell growth at 80 mM TDG. *A. xylosoxidans* was applied to degrade TDG in batch and continuous stirred-tank reactors, and a reasonable biomineralization efficiency was observed (7).

Several bacteria are known to possess C-P cleaving activity: the *Pseudomonas*, *Enterobacter*, *Escherichia*, *Arthrobacter*, and *Streptomyces* species (8–12). Three C-P-cleaving enzymes with different substrate specificities

have been isolated and characterized: phosphonate (13), C-P lyase (14), and phosphonoacetate hydrolase (15). However, note that all the experiments on biodegradation of the target C-S and C-P compounds have been performed under aerobic conditions. To our knowledge, the data on anaerobic biodegradation of these compounds are absent in the literature, but such information is quite important in order to know the fate of hydrolysis products that will be inevitably released from bituminized blocks into landfill environments. Thus, the present study is devoted to the assessment of the toxicity and biodegradability of the hydrolysis products of chemical warfare agents under methanogenic conditions.

## Materials and Methods

### *Inoculum, Medium, and Batch Vessels*

Sludge from the laboratory upflow anaerobic sludge blanket reactor treating liquid hen manure fraction was used as inoculum (16). The acetoclastic methanogenic activity of the sludge was around 0.4 g of chemical oxygen demand (COD) (g of volatile suspended solids [VSS] · d).

The basal medium contained the following: 280 mg/L  $\text{NH}_4\text{Cl}$ ; 10 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; 250 mg/L  $\text{K}_2\text{HPO}_4$ ; 100 mg/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.0 mg/L EDTA; 0.2 mg/L resazurin; 5000 mg/L  $\text{NaHCO}_3$ ; 0.05 mg/L  $\text{H}_3\text{BO}_3$ ; 2.0 mg/L  $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ ; 0.05 mg/L  $\text{ZnCl}_2$ ; 0.05 mg/L  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 0.03 mg/L  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ; 2.0 mg/L  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ; 0.05 mg/L  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.1 mg/L  $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ ; pH 7.2.

The experiments were performed in 120-mL glass flasks in a batch regime at 30°C without stirring. The sludge (1 mL per flask, final concentration around 1 g of VSS/L) was added to the flasks with the basal medium (final volume of a liquid phase was 25 mL). The flasks were then sealed with rubber stoppers, flushed with inert gas (argon), and incubated overnight to deplete all readily biodegradable organic compounds introduced with the seed sludge.

### *Toxicity Assay*

The toxicity of the target compounds at concentrations of 0.5, 1.0, 2.0, 5.0, 7.5, and 10.0 g/L was studied with regard to their influence on acetoclastic methanogenic activity of anaerobic sludge. Two types of experiments were carried out. In the first case, the sludge activity was determined in the presence of a toxicant. For this purpose, after a 24-h sludge starvation in the basal medium, an investigated toxic compound was added to the flasks that were then incubated for another 24 h. Thereafter, 1 mL of sodium acetate solution (final concentration 2 g of COD/L) was added in the same flasks without removal of toxicant, and the flasks were flushed with argon. In the second set of experiments, after a 24-h incubation with a toxicant, the liquid phase was decanted and the sludge was quickly washed by an oxygen-free mineral medium. Immediately after this procedure, a new portion of a toxicant-free medium containing 2 g of

Table 1  
Physicochemical Properties of Hydrolysis Products  
of Chemical Warfare Agents<sup>a</sup>

Compound	Physical state (25°C, 1 atm)	Molecular mass (Da)	Boiling point (°C)	Solubility in water/ diethyl ether
Ethanolamine (18)	Liquid	61	171	∞/weak
TDG (18)	Liquid	122	168	∞/weak
Mercaptoethanol (19)	Liquid	78	157–158	Good/good
MPhA (20)	Solid	96	105–107 <sup>b</sup>	Good/weak
Diisobutyl ester of MphA (20)	Liquid	208	115–116	Weak/good

<sup>a</sup>See refs. 18–20.

<sup>b</sup>Melting point.

COD/L of sodium acetate was added and the flasks were flushed with argon. In both cases, the concentration of CH<sub>4</sub> in the headspace was recorded throughout the incubation, and specific acetoclastic activities of the sludge were calculated from a linear segment of kinetic curves of methane production. The acetoclastic activity of the sludge not treated with a toxicant was taken as a control. The IC<sub>50</sub> values for the investigated compounds were estimated from the concentration dependencies of the specific activities. All experiments were carried out in triplicate, and all the data presented in Tables 1–4 and Figs. 1 and 2 are the mean of triplicates.

#### Anaerobic Biodegradability Assay

In the first set of biodegradability trials, the tested compounds were used as a sole carbon source. For this purpose, after a 24-h starvation of the sludge in the basal medium, 1 mL of a concentrated target substrate solution was added to the flasks, which were then flushed with argon. MPhA was neutralized before addition into the medium. The concentration of the examined substrates was 1 g/L.

In the second set of experiments, glucose or a mixture of propionic and butyric acids (1:1, v/v) were used as cosubstrates to stimulate an anaerobic biodegradation of the hydrolysis products with C-S and C-P bonds. The cosubstrates were simultaneously added with the main substrate in the ratio of 1:1 (as COD). The total (sum of substrate and cosubstrate) COD concentrations were 0.5 and 2 g/L for the S- and P-containing substrates, respectively.

CH<sub>4</sub>, H<sub>2</sub>, and CO<sub>2</sub> in the gas phase as well as volatile fatty acids (VFAs) and alcohols in the liquid phase were monitored during all the experiments.

#### Analysis

The CH<sub>4</sub>, H<sub>2</sub>, and CO<sub>2</sub> content in the headspace of the flasks was determined on a gas chromatograph LHM 8MD (Yagat, Moscow, Russia)

Table 2  
Relative Aceticlastic Activity (%) of Sludge in the Presence (1) and After Removal (2) of Toxicant<sup>a</sup>

Concentration (g/L)	Ethanolamine		TDG		Mercaptoethanol		MPhA		Diisobutyl ester of MPhA	
	1	2	1	2	1	2	1	2	1	2
0.0	100.0 ± 8	100 ± 14	100 ± 10	100 ± 3	100.0 ± 6	100 ± 8	100 ± 28	100 ± 25	100 ± 6	100 ± 14
0.5	100.0 ± 3	112 ± 2	102 ± 13	98 ± 4	76.0 ± 26	76 ± 17	100 ± 12	100 ± 7	83 ± 3	83 ± 3
1.0	12.0 ± 4.7	53 ± 10	93 ± 6	95 ± 3	69.0 ± 8	69 ± 5	100 ± 29	100 ± 34	67 ± 2	62 ± 0.6
2.0	1.3 ± 0.3	20 ± 9	78 ± 1	85 ± 2	46.0 ± 1	62 ± 6	100 ± 22	100 ± 29	29 ± 1.5	28 ± 0.3
5.0	0.0	0.0	32 ± 6	46 ± 6	36.0 ± 8	49 ± 8	100 ± 6.3	100 ± 14	0.0	0.0
7.5	0.0	0.0	30 ± 9	51 ± 7	18.5 ± 6	47 ± 6	100 ± 22	100 ± 21	0.0	0.0
10.0	0.0	0.0	4 ± 2	13 ± 1	1.5 ± 0.1	39 ± 4	100 ± 12	100 ± 12	0.0	0.0

<sup>a</sup>The activity of the sludge not treated with a toxicant was taken as 100%.

Table 3  
Anaerobic Biodegradability of the Hydrolysis Products  
of Chemical Warfare Agents

Compound	Biodegradability (%)	Duration of incubation (d)	Toxicity (IC <sub>50</sub> ) (g/L)	Lag period (d)
Ethylene glycol	100	6	Not toxic (22)	2
Isopropanol	100	13	Not toxic (21)	0
Isobutyl alcohol	100	18	Not toxic (21)	0
Ethanolamine	100	22	0.63 <sup>a</sup>	8
TDG	42	185	4.2 <sup>a</sup>	52
Mercaptoethanol	29	55	2.8 <sup>a</sup>	0
MPhA	0	152	Not toxic <sup>a</sup>	—
Diisobutyl ester of MPhA	0	152	1.39 <sup>a</sup>	—

<sup>a</sup>Determined from the data of columns 1 of Table 1.

Table 4  
Anaerobic Biodegradability of the Hydrolysis Products  
of Chemical Warfare Agents in the Presence of Cosubstrates

Substrate (g of COD/L)	Cosubstrates (g of COD/L)	Duration of incubation (d)	Anaerobic biodegradability (%)
TDG			
0.5	None	55	31
0.25	Glucose (0.25)	25	48
0.25	VFA (0.25)	32	100
Mercaptoethanol			
0.5	None	55	29
0.25	Glucose (0.25)	55	0
0.25	VFA (0.25)	55	0
MPhA			
1.0	None	152	0
1.0	Glucose (1.0)	152	0
1.0	VFA (1.0)	152	0
Diisobutyl ester of MPhA			
1.0	None	152	0
1.0	Glucose (1.0)	152	0
1.0	VFA (1.0)	152	0

equipped with a steel column (2 m × 2 mm) packed with Porapak QS (80/100 mesh, Serva Feinbiochemica, Heidelberg/New York). The temperature of the column, injector port, and conductivity detector was set at 50°C. Argon was used as a carrier gas with a flow rate of 30 mL/min.

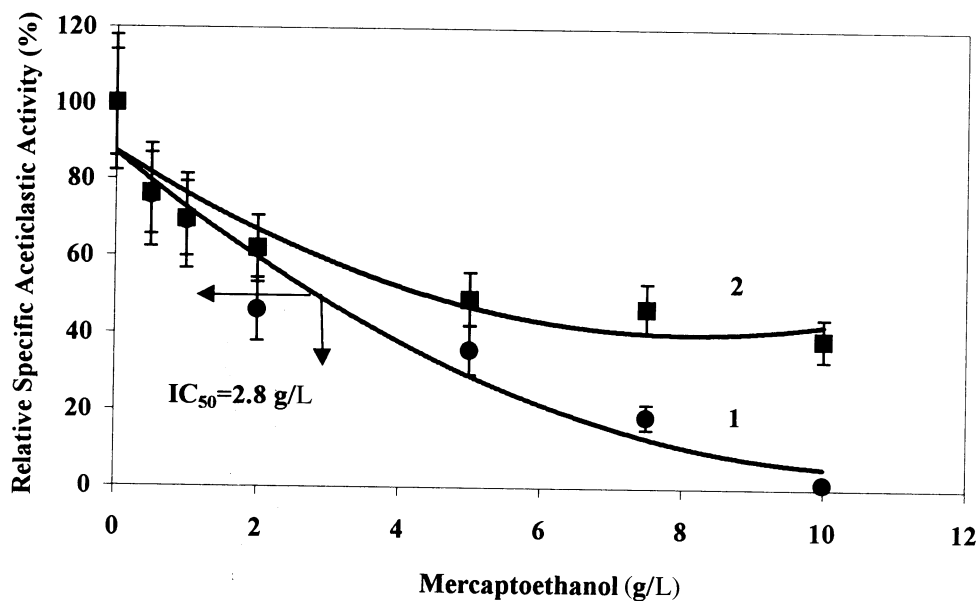


Fig. 1. Relative aceticlastic activity of the sludge in the presence (1) and after removal (2) of mercaptoethanol (the activity of the sludge not treated with mercaptoethanol was taken as 100%).

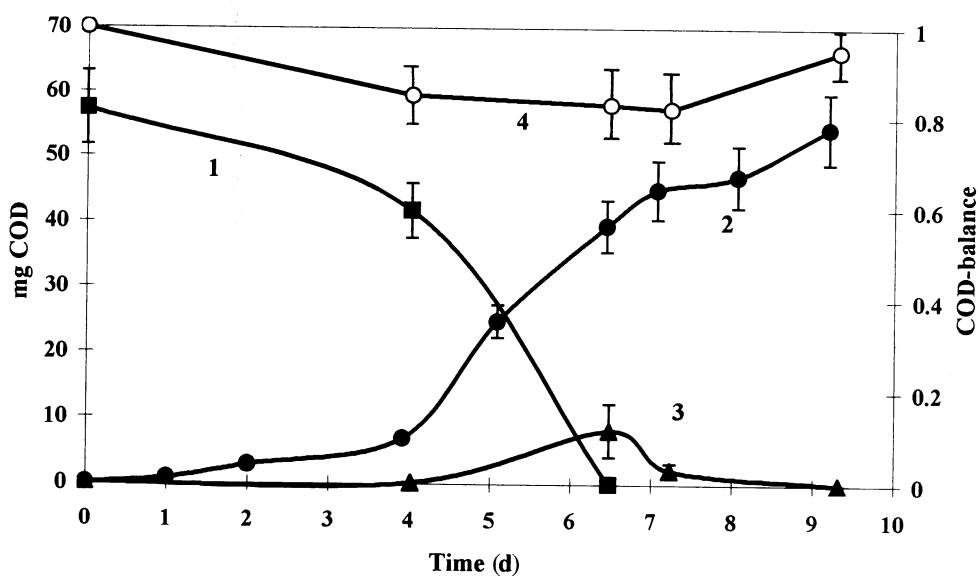


Fig. 2. Kinetics of ethylene glycol biodegradation under anaerobic conditions (initial quantity of ethylene glycol, 57.4 mg of COD; sludge concentration, 1 g of VSS/L; pH 7.0; 30°C). 1, ethylen glycol; 2, methane; 3, acetic acid; 4, COD balance.

VFAs and alcohols were determined on a gas chromatograph Chrom-5 (Laboratorni Pristroje, Praha, Czechoslovakia) equipped with a glass column (2 m × 2 mm) packed with Porapak QS (80/100 mesh). The tempera-

ture of the column, injector port, and flame ionization detector was set at 190, 200, and 200°C, respectively. Argon was used as a carrier gas with a flow rate of 30 mL/min.

Total suspended solids and VSS were determined as described in ref. 17. pH was measured using a Jenco electronics pH meter (Jenco Electronics, Ltd., Taipei, Taiwan).

### Calculations

The acetoclastic methanogenic activity of the sludge was calculated from the slope of the linear segment of cumulative methane concentration line (% CH<sub>4</sub>/h) in the gas phase, according to the following formula:

$$\text{ACT} = (S/100) \cdot 24 \cdot V \cdot f_{\text{CH}_4} \cdot (1/\text{VSS}) \quad (4)$$

where ACT is the specific methanogenic activity (CH<sub>4</sub> g of COD/[g of VSS · d]), *S* is the slope of cumulative CH<sub>4</sub> concentration line (%/h), *V* is the volume of headspace, *f*<sub>CH<sub>4</sub></sub> is the conversion factor from liters of CH<sub>4</sub> to grams of COD (g/L), VSS is the volatile suspended solids (g), and *f*<sub>CH<sub>4</sub></sub> is equal to 2.57 g of COD/L at 30°C.

### Chemicals

MPhA and diisobutyl ester of MPhA (95% purity) were synthesized by L. V. Kovalenko, and TDG (95% purity) by T. S. Serebryakova and A. L. Chemishkyan (Department of Chemistry and Technology of Organic Synthesis, D. I. Mendeleev Russian Chemico-Technological University, Moscow). All other chemicals (Reachim, Moscow, Russia) were of the highest purity available and were used without further purification. Table 1 presents some physicochemical properties of the target compounds.

## Results and Discussion

### Toxicity

Since it was previously shown that isopropanol and isobutyl alcohol in a concentration of about 4 g/L (21) and ethylene glycol in a concentration up to 10 g/L (22) were not toxic and easily degradable by anaerobic methanogenic sludge, a toxicity was assessed only for five principal compounds of detoxification of chemical warfare agents: ethanolamine, TDG, mercaptoethanol, MPhA, and diisobutyl ester of MPhA. Table 1 summarizes the results of their influence on acetoclastic activity of the sludge. It can be seen that under the presence of toxicant (columns 1 of Table 2), only MPhA had no influence on the acetoclastic activity in a concentration up to 10 g/L, whereas all other compounds manifested a strong inhibition of methane production (note the IC<sub>50</sub> values from Table 3). Ethanolamine and diisobutyl ester of MPhA were shown to be the most toxic compounds tested; methane formation was completely inhibited at a concentration of 5 g/L (Table 2). The different toxic effects of MPhA and its ester can be



attributed to a difference in their hydrophobicity. A correlation between hydrophobicity and methanogenic toxicity was demonstrated for the various homologous series of xenobiotic compounds (23).

To investigate whether the inhibition of methanogenic activity resulted from a toxicant action was reversible, the acetoclastic activity of the sludge was also assessed after a 24-h exposure to the toxicant followed by removal of the latter (Table 2, columns 2). It is seen that only the action of diisobutyl ester of MPhA was irreversible, whereas removal of ethanolamine, TDG, and mercaptoethanol resulted in a partial restoration of the sludge activity. This effect was the most pronounced for mercaptoethanol (Fig. 1).

### *Biodegradability*

Table 3 summarizes the results of the biodegradability trials for eight compounds tested. With regard to the behavior of anaerobic biodegradability, the tested compounds can be divided into three groups. The substrates of the first group (ethylene glycol, isopropanol, isobutyl alcohol, and ethanolamine) are characterized by a complete and relatively fast biomineralization even by unadapted sludge. These observations are in accordance with literature data (21,22). Figure 2 represents typical kinetics of methanogenic degradation of the substrates of this group. It can be seen that the COD balance in the system on the basis of measured components varied between 85 and 100%.

The substrates of the second group (sulfur-containing compounds: TDG and mercaptoethanol) are slowly biodegraded, whereas the substrates of the third group (phosphorus-containing compounds: MPhA and its diisobutyl ester) seem not to be biodegradable under methanogenic conditions.

### *Influence of Addition of Cosubstrates on Biodegradability*

Easily biodegradable cosubstrates (glucose, alcohols, VFA, and so forth) are often used to enhance the anaerobic biodegradation of recalcitrant xenobiotics (24). Table 4 presents the generalized results of influence of such stimulation on biodegradability of substrates of the second and third groups. As one can see, the addition of cosubstrate was effective only for the biodegradation of TDG. In the presence of VFA, TDG was completely mineralized within 32 d with a short (if any) lag period observed. For comparison, the duration of lag period when TDG was used solely was almost as long as 2 mo (Table 3). But, when glucose was used as a cosubstrate, TDG was readily converted by 48% during 25 d, and the lag period was also practically absent. However, further continuation of incubation did not result in further mineralization of TDG. For other substrates, the presence of cosubstrates did not have any positive influence on biodegradation. Moreover, mercaptoethanol, being biodegradable without cosubstrate (Table 3), was not mineralized at all in the presence of both a VFA mixture and glucose. These observations, as well as an incomplete

biomineralization of TDG in the presence of glucose, need further investigation focused on the preferential use of C-source in such anaerobic bacterial systems.

As we have shown, no degradation of the phosphorus-containing substrates was observed under imposed methanogenic conditions with or without cosubstrate. It is likely that  $\text{CO}_2$  is too weak an electron acceptor to promote decomposition of these substrates. Thus, the other electron acceptors (nitrate, sulfate, and so on) and anaerobic conditions (e.g., sulfate reducing, denitrifying) should also be investigated to obtain information about the fate of MPhA-derived compounds under their release into the environment. If one aims to fulfill a complete mineralization of the hydrolysis products with C-P bonds, a possible solution could also be a combination of aerobic and anaerobic processes for the complete biodegradation of this class of xenobiotics.

## Conclusion

Among the investigated hydrolysis products of chemical warfare agents, only MPhA does not have any toxic effect with regard to the acetoclastic methanogenic activity. The toxicity of other compounds varied between moderate (TDG, mercaptoethanol) to strong (ethanolamine, diisobutyl ester of MPhA). From the point of view of biodegradability, the data obtained in this study show that all the products of chemical detoxification of mustard gas (ethanolamine, ethylene glycol, TDG, mercaptoethanol) can be biomineralized under methanogenic conditions, so they will hardly accumulate in the environment. On the contrary, phosphorus-containing compounds from the chemical detoxification of nerve warfare agents (Sarin, Soman,  $\text{V}_x$ -gases) are quite persistent under these conditions, and their fate in anaerobic environments needs further investigation.

## Acknowledgments

This work was partially supported by the Russian Ministry of Science and Technology New Methods of Bioengineering Program (grant no. 7-150). The authors are grateful to T. S. Serebryakova, L. V. Kovalenko, and A. L. Chemishkyan for the synthesis of sulfur- and phosphorus-containing compounds.

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