Hydrolysis of Terbufos Using Simulated Environmental Conditions: Rates, Mechanisms, and Product Analysis

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This study focuses on the hydrolysis of terbufos, an organophosphorus pesticide. Combining GC-MS and wet chemistry methods, di-*tert*-butyl disulfide and formaldehyde were identified and quantified as major degradation products. Diethyl dithiophosphate was also indirectly identified as a degradation product under alkaline conditions. Hydrolysis rate constants of terbufos under homogeneous conditions were comparable to those of phorate and show relative insensitivity to pH under slightly acidic to neutral pH conditions, as the observed rate constants varied only in the range of $(4.5-5.0) \times 10^{-6}\ s^{-1}$ between pH 5.7 and 9.4; neutral hydrolysis is thus the most dominant hydrolysis pathway of terbufos in ambient waters. The mechanisms for terbufos hydrolysis and the formation of the major products and their temporal profiles are discussed. To assess the environmental impact of degradation products of this widely used pesticide, Microtox was used to analyze the toxicity of terbufos and two of its degradation products: diethyl dithiophosphate and di-*tert*-butyl disulfide; the EC_{50} of terbufos was found to be $>17\ \mu M$, whereas the EC_{50} of di-*tert*-butyl disulfide was $1.3\ \mu M$.

Keywords: *Nucleophilic attack; di-tert-butyl disulfide; formaldehyde; O,O,S-triethyl phosphorodithioate; Microtox; toxicity*

INTRODUCTION

Many esters and thioesters of phosphoric and thiophosphoric acid (Figure 1a) are used as pesticides for their inhibitory activity toward cholinesterase, a key enzyme involved in the metabolism of acetylcholine (one of the major neuron transmitters) (*1*). Because cholinesterase is a rather common enzyme among animals that have a neural system, these pesticides can actually cause harm to many nontarget species. With one of the strongest agricultural industries in the world, the United States is a major user of organophosphorus pesticides (*2*). In recent years, the U.S. Environmental Protection Agency has begun to consider tougher regulations regarding the use of organophosphorus compounds. In the meantime, the threat to environmental quality, partly due to the toxic impact of anthropogenic compounds on wildlife, has resulted in active research on the role of commonly used pesticides, many of which belong to the organophosphorus family (*3*−*5*). Several organophosphorus pesticides and their degradation products have already been shown to be deleterious to aquatic fauna (*6*, *7*).

Oxidation, photolysis, and hydrolysis are considered to be the three main degradation pathways for organophosphorus pesticides (*8*−*10*). Three hydrolysis pathways for these compounds have been proposed, in which acid, base, or neutral species serve as the catalysts or nucleophiles (*9*, *11*). These pathways are essentially nucleophilic attacks; the target atom is either the central P atom or the C atom in the ester side chain, although the so-called acid-catalyzed pathway usually starts with the protonation of a functional group in the ester side chain. The relative significance of the C−O or C−S cleavage versus P−O or P−S cleavage depends on the hydrolysis temperature and the nature of the leaving group during the hydrolytic nucleophilic substitution. The contribution of each of the three mechanisms to the total hydrolysis is pH-dependent, because the concentrations of the major nucleophiles or catalysts (H^+ and OH^-) are pH-dependent. The intermediates (i.e., phosphodiesters and phosphomonoesters) can undergo further hydrolysis, although they react at much slower rates (*9*).

Terbufos (CAS Registry No. 13071-79-9) belongs to the phosphorodithioates (Figure 1b), a subgroup of organophosphorus compounds. After more than two decades since its introduction, it is still used extensively in the United States as well as in several Latin American countries as a soil pesticide; it targets root pests that feed on corn, sugar beets, banana, etc. Although terbufos was chosen as one of the extremely hazardous substances on the Superfund list (Section 302 of EPCRA) as well as a candidate for the Draft Drinking Water Contaminant List following the Safe Drinking Water Act (1997), the United States alone consumed 4.5 millions pounds of terbufos on corn in 1996 (*12*). Terbufos can be oxidized to terbufos sulfoxide, terbufos sulfone, terbufos oxon, and its sulfoxide and sulfone in soil or within plants (*13*, *14*). Terbufos is also often detected in agricultural surface runoff waters (*15*), but studies on its hydrolysis under simulated environmental conditions have been limited (*16*, *17*). In particular, studies focusing on its hydrolysis products and path-

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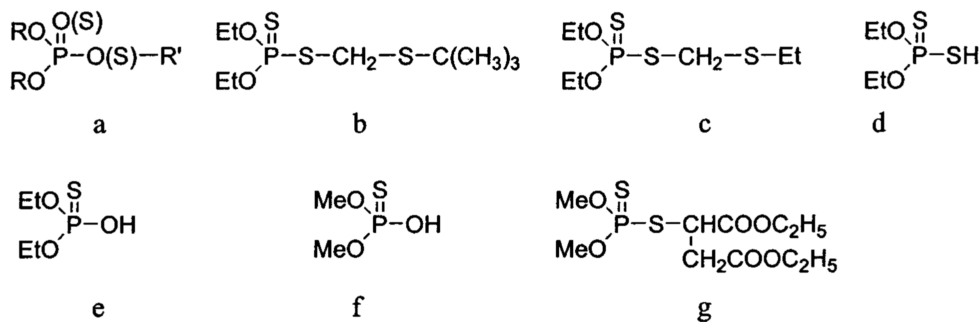

Figure 1. (a) Organophosphorus esters; (b) terbufos; (c) phorate; (d) *O,O*-diethyl dithiophosphate; (e) *O,O*-diethyl phosphorothionic acid; (f) *O,O*-dimethyl phosphorothionic acid; (g) malathion.

ways are lacking. Furthermore, although literature on the toxicity of terbufos and its oxidative metabolites abounds (*18*, *19*), the toxicity of its hydrolysis products has not been sufficiently addressed. The current study is intended for the evaluation of the hydrolytic fate and ecological impact of terbufos under abiotic conditions. Buffers of relatively low concentration (<0.01 M) were used in most experiments to better reflect natural conditions, and selected experiments were designed to assess the effect of buffer concentration on the hydrolysis rate.

MATERIALS AND METHODS

Hydrolysis Experiments. Boric acid, NaOH, NaOAc, methanol, benzene, CCl_4, and HNO_3 were all obtained from Fisher Scientific (Pittsburgh, PA). Neat terbufos (98%) was purchased from Chem Service (West Chester, PA). An acetone stock solution of terbufos (1:100 v/v) was stored at 4 °C. Hydrolysis reactors were Teflon bottles (130 or 260 mL) (Fisher Scientific), wrapped in aluminum foil. The conditions for homogeneous hydrolysis were mostly the same as described previously (*20*). Additionally, homogeneous hydrolysis of terbufos was carried out at pH 4.7 (in 0.01 M acetic acid/acetate buffer), pH 9.4 and 10.25 (in 0.01 M boric acid/borate buffer). Carbon tetrachloride (0.1 mL) was added to all reactors as a biocide. To investigate the effect of the buffer concentration on the hydrolysis rate of terbufos, hydrolysis reactions were carried out at 0.003, 0.006, and 0.01 M for both acetic acid/acetate buffer (pH 5.7) and boric acid/borate buffer (pH 8.5) and also at 0.014 M for boric acid/borate buffer (pH 8.5). Hydrolysis was carried out at 25−28 °C, unless otherwise noted. The total volume of the reaction mixture was kept at 260 or 130 mL, so that very little headspace was left, and the starting concentration of terbufos was 17 μM. The duration of the hydrolysis experiments was 8−14 days, during which seven to eight aliquots of 5 or 10 mL were withdrawn into amber vials. The above procedure allowed the hydrolysis to proceed over two to three half-lives of terbufos. Amounts of 5 or 10 mL of the corresponding buffer were added back to the reaction mixture after each sample withdrawal (the dilution factor is considered in the calculation of k_{obs}), and the pH was adjusted back to the starting value (normally the pH drift was <0.1) with NaOH or HNO_3.

GC and GC-MS Analysis. GC samples were analyzed on a Shimadzu GC 17A version 3 equipped with an FPD detector and a Shimadzu AOC-20s autosampler. GC samples were prepared by extracting 5 volumes of a hydrolysis sample with 1 volume of benzene, shaken for 2 min, and stored in the refrigerator until analysis. Detailed GC conditions were as follows: a 30-m, 0.53 mm i.d. fused silica capillary column with 1.5-μm film thickness (ZB-1, Zebron) and carrier gas of helium (33 kPa); splitless mode with P filter for terbufos and S filter for the disulfide analyses; initial temperature, 105 °C for 2 min; temperature ramp, increased at 17 °C/min to 250 °C, held for 3 min; injector port temperature, 250 °C; FPD detector temperature, head = 190 °C and base = 300 °C; oven equilibration time, 3 min. GC standards of terbufos and di-*tert*-butyl disulfide were prepared at 17, 8.5, 4.3, 2.1, and 1.1 μM, after which a calibration curve for each was obtained. Every other sample was injected twice to monitor reproducibility, which was found to be acceptable (<15% RSD).

Additional GC analyses were carried out on an HP 5890 series II GC with an FID detector (Hewlett-Packard Co., Palo Alto, CA). Di-*tert*-butyl disulfide was obtained from Aldrich Chemical Co. (Milwaukee, WI). Five volumes of a hydrolysis sample was mixed with 1 volume of benzene containing 50 mg/L 4-chloro-3-methylphenol (internal standard for GC; Aldrich Chemical Co.), shaken for 2 min, and then stored at 4 °C prior to analysis. GC conditions were as described (*20*), except that the column temperature was increased at 17 °C/min. GC standards for terbufos were prepared like the hydrolysis samples with aqueous terbufos concentrations of 3.5, 6.9, 17, and 35 μM prior to benzene extraction. The aqueous concentrations of di-*tert*-butyl disulfide in GC standards were 1.7, 3.5, 8.7, and 17 μM prior to benzene extraction. Every other sample was injected twice during GC analysis to monitor the reproducibility.

An HP 5890 series II GC with HP 5970 MS detector (Hewlett-Packard Co.) was used for GC-MS analysis. GC-MS conditions were the same as described previously (*20*). Terbufos hydrolysis samples were extracted with Empore SDB-RPS extraction disks (Fisher Scientific), which were then eluted with 10 mL of methylene chloride (Fisher Scientific) each. The manufacturer's (3M Inc., St. Paul, MN) protocol was followed for the procedure except that acetonitrile was used instead of methanol to condition the extraction disks, because methanol can result in transesterification of terbufos. The eluates served as GC-MS samples. Methylene chloride solutions of pure compounds were injected as standards whenever possible during the product analysis.

Product Analysis. The Hantzsch reaction was used to quantify HCHO (*21*). HCHO (Fisher Scientific) reacts with ammonium acetate (Fisher Scientific) and acetylacetone (Aldrich Chemical Co.) to form 3,5-diacetyl-1,4-dihydrolutidin, the absorbance of which at 415 nm was measured with a Shimadzu UV-1201S spectrophotometer (Shimadzu Scientific Instruments, Inc.) using 1- and 5-cm quartz cuvettes. Nash reagent [30% NH_4OAc (w/v) and 0.4% acetylacetone (v/v) in water] and formaldehyde standard solutions were prepared de novo as described in Smith and Erhardt (*22*). Doubly deionized H_2O replaced the formaldehyde solution in the blank. To study the temporal profile of HCHO formation during terbufos hydrolysis under deoxygenated conditions, terbufos hydrolysis mixture (pH 8.5, $[\text{terbufos}]_{init}$ = 17 μM) samples were prepared as follows: six parallel hydrolysis reactions were set up, and the solutions were purged with high-purity N_2 for 20 min prior to the addition of terbufos to reduce the dissolved oxygen to a negligible level. The reactions were stopped after 1, 2, 4, 5, 10, and 12 days, respectively, at which times 5-mL samples were withdrawn and analyzed for HCHO concentration immediately. Additional hydrolysis reactions were set up (without N_2 purging), similarly to the aforementioned terbufos hydrolysis experiments to study HCHO production in the presence of dissolved oxygen. In these reactions,

Table 1. Hydrolysis Rate Constants of Terbufos

reaction conditions[a]	$k_{obs} \pm SD^b$ (s^{-1})	$t_{1/2}$ (h)	no. of replicates
pH 4.7 with acetic acid/acetate buffer	$3.6 \times 10^{-6} \pm 3.5 \times 10^{-7}$	54	2
pH 5.7 with acetic acid/acetate buffer	$4.5 \times 10^{-6} \pm 3.8 \times 10^{-7}$	42	4
pH 8.5 with boric acid/borate buffer	$5.0 \times 10^{-6} \pm 3.3 \times 10^{-7}$	39	2
pH 9.4 with boric acid/borate buffer	$4.6 \times 10^{-6} \pm 2.6 \times 10^{-7}$	42	2
pH 10.25 with boric acid/borate buffer	$7.2 \times 10^{-6} \pm 5.8 \times 10^{-8}$	27	2

[a] Initial terbufos concentration of 17 μM. [b] SD, standard deviation.

5 mL of the reaction solution was taken out at each sampling time (the dilution factor is included in the HCHO production data), and 5 mL of the buffer was added back to the reactor.

For a qualitative determination of H_2S formation, a terbufos hydrolysis experiment at pH 8.5 was set up and purged with high-purity N_2 for 20 min to remove dissolved oxygen. A syringe through a rubber septum was used to withdraw 20-mL aliquots, and the liquid volume was replaced by the N_2 stored in a balloon that was connected to the reactor via a needle through the septum. Samples were quickly mixed with an equal volume of a preservation buffer (i.e., 2 M NaOH, 0.2 M H_2Na_2EDTA, and 0.2 M ascorbic acid in water) and stored at 4 °C. Sulfide concentrations of the samples were then analyzed by a model 94-16 silver/sulfide electrode from Orion Research Inc. (Boston, MA) following the manufacturer's protocol.

To facilitate the detection of any potential polar and acidic terbufos hydrolysis products by GC-MS, methylation with diazomethane was carried out for a terbufos hydrolysis mixture at pH 8.5. Diazomethane was synthesized de novo using the MNNG diazomethane generation apparatus (Aldrich Chemical Co.), and the manufacturer's protocol was followed. After shaking of the hydrolysis mixture and the diethyl ether layer containing the diazomethane, the diethyl ether layer was used as the GC-MS sample following phase separation. Neat *O,O*-diethyl dithiophosphate (Aldrich Chemical Co.) (Figure 1d) was also similarly methylated to serve as a GC-MS standard.

Toxicity Analysis. Microtox from Azur Environmental Co. (Carlsbad, CA) was used to measure the toxicity of terbufos and two of its identified hydrolysis products: di-*tert*-butyl disulfide and *O,O*-diethyl dithiophosphate. The initial concentration of all samples was 17 μM. A phenol solution at 100 μM was used as the toxicity standard, as recommended by the manufacturer. A series of homogeneous hydrolysis reactions were set up, and the temporal profile of toxicity of the reaction mixtures was monitored for ~10 days to support the proposed hydrolysis mechanism in terms of toxicity. The aforementioned dilution factor was also considered for the acquired Microtox data. A basic test procedure from the manufacturer's manual was used, and the assay was repeated three times for all samples.

RESULTS AND DISCUSSION

Hydrolysis Experiments. The hydrolysis of terbufos can be treated as a pseudo-first-order reaction in buffered solutions of constant pH; hence, the following equation applies: $dC/dt = -k_{obs}C$. The observed rate constant for the hydrolysis reaction, k_{obs}, is assumed to be the sum of three terms: $k_{obs} = k_a[H^+] + k_N + k_b[OH^-]$, where k_a, k_b, and k_N represent the rate constants for acid-catalyzed, base-catalyzed, and neutral hydrolysis pathways, respectively (*9*). When ln [terbufos] versus reaction time is plotted (Figure 2), k_{obs} is equal to the slope. The kinetic data of terbufos hydrolysis display good fits with a pseudo-first-order reaction model, and the observed hydrolysis rate constants of terbufos at five pH values (4.7, 5.7, 8.5, 9.4, and 10.25) are shown in Table 1. The results agree very well with those of Chapman and Cole (*17*) and indicate that pH has a very small impact on the hydrolysis rate of terbufos. This is due to the dominant influence of neutral hydrolysis of terbufos (i.e., k_N in the aforementioned equation). Furthermore, the data in Table 1 indicate that the magnitude of k_a is quite small and acid-catalyzed hydrolysis should not play an important role for terbufos hydrolysis. It can also be seen that the hydrolysis kinetics of terbufos is similar to that of phorate (*20*), which is not surprising considering their structural similarities (Figure 1). The effect of the buffer concentration on terbufos hydrolysis rates is shown in Table 2.

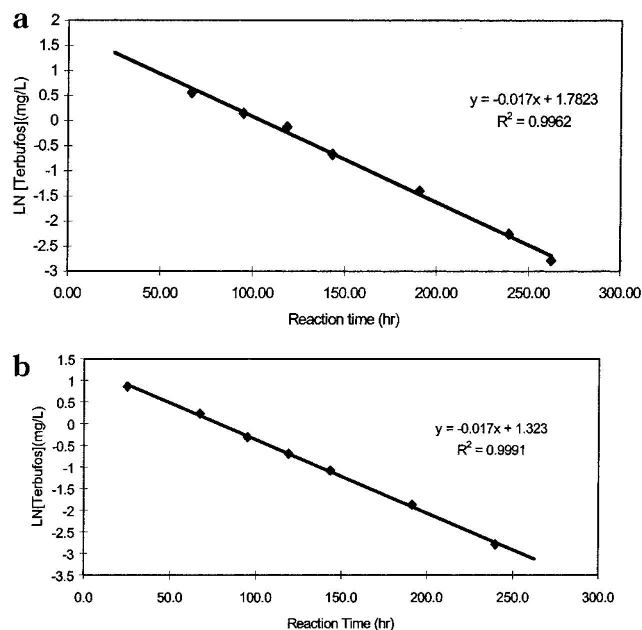

Figure 2. Pseudo-first-order kinetics of terbufos hydrolysis: (a) pH 5.7; (b) pH 8.5.

Table 2. Terbufos (at 17 μM) Hydrolysis Rate Constants Reflecting the Effect of Buffer Concentration

pH	buffer concn[a] (M)	av temp (°C)	$k_{obs} \pm SD^b$ (s^{-1})	$t_{1/2}$ (h)	no. of replicates
8.5	0.003	26.9	$8.0 \times 10^{-6} \pm 9.7 \times 10^{-7}$	24	3
8.5	0.01	27.1	$7.3 \times 10^{-6} \pm 8.9 \times 10^{-7}$	26	3
8.5	0.003	28.5	$1.1 \times 10^{-5} \pm 2.0 \times 10^{-8}$	17	2
8.5	0.006	28.5	$1.0 \times 10^{-5} \pm 3.8 \times 10^{-7}$	19	2
8.5	0.01	28.0	$8.5 \times 10^{-6} \pm 1.1 \times 10^{-7}$	23	2
8.5	0.014	27.9	$8.5 \times 10^{-6} \pm 1.6 \times 10^{-6}$	23	3
5.7	0.003	26.9	$8.0 \times 10^{-6} \pm 9.7 \times 10^{-7}$	26	3
5.7	0.01	26.3	$5.7 \times 10^{-6} \pm 4.2 \times 10^{-7}$	34	2
5.7	0.003	28.5	$1.0 \times 10^{-5} \pm 2.8 \times 10^{-7}$	19	2
5.7	0.006	28.4	$9.5 \times 10^{-6} \pm 6.0 \times 10^{-7}$	20	2
5.7	0.01	28.0	$6.0 \times 10^{-6} \pm 6.5 \times 10^{-7}$	32	3

[a] pH 5.7 with acetic acid/acetate buffer, pH 8.5 with boric acid/borate buffer. [b] SD, standard deviation.

At 26.9 °C, the half-lives of terbufos in 0.003 M buffer solutions are 24 h at pH 8.5 and 26 h at pH 5.7, which are longer in comparison to those at a temperature of 34.5 °C (i.e., 12 h at pH 8.5 and 14 h at pH 5.7). These results (Table 3) clearly indicate that the terbufos

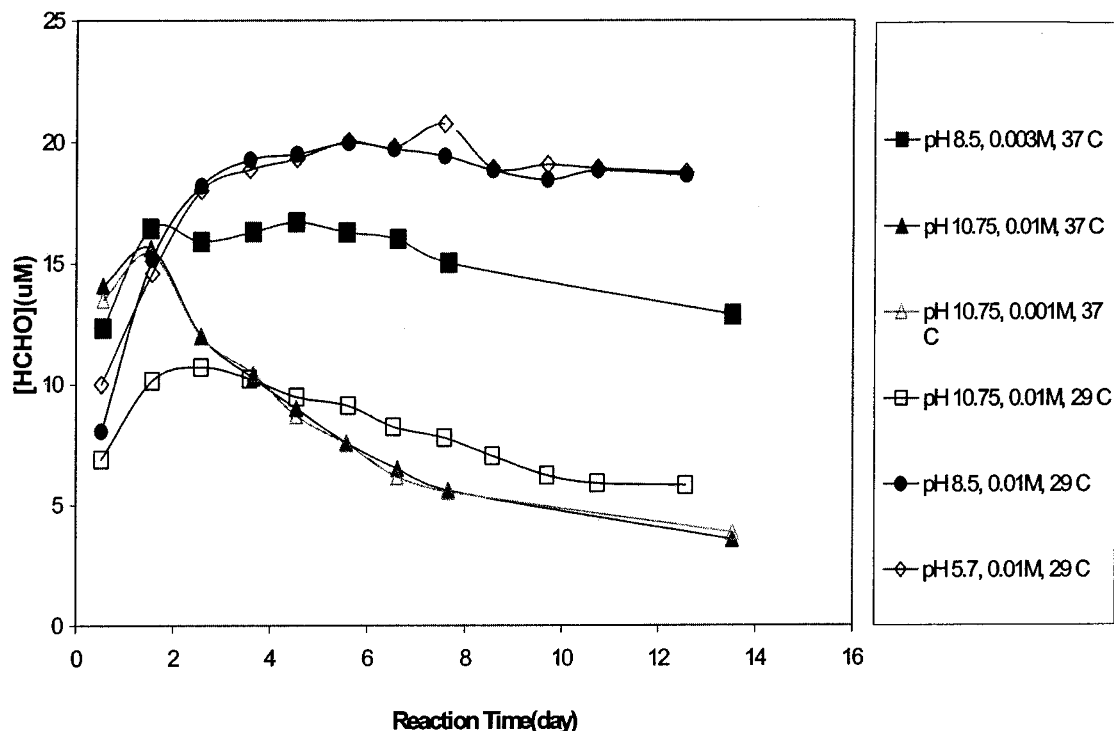

Figure 3. Time course of HCHO formation from terbufos hydrolysis at 29 and 37 °C.

Table 3. Effect of Temperature on 17 μM Terbufos Hydrolysis

buffer concn[a] (M)	pH	av temp (°C)	k_{obs} ± SD[b] (s^{-1})	$t_{1/2}$ (h)	no. of replicates
0.003	8.5	26.9	$8.0 \times 10^{-6} \pm 9.7 \times 10^{-7}$	24	3
		28.5	$1.1 \times 10^{-5} \pm 2.0 \times 10^{-7}$	18	2
		34.5	$1.6 \times 10^{-5} \pm 1.5 \times 10^{-6}$	12	2
0.003	5.7	26.9	$7.4 \times 10^{-6} \pm 1.1 \times 10^{-6}$	26	3
		28.5	$1.0 \times 10^{-5} \pm 2.4 \times 10^{-7}$	19	2
		34.5	$1.4 \times 10^{-5} \pm 1.2 \times 10^{-6}$	14	2
0.006	8.5	26.8	$9.8 \times 10^{-6} \pm 1.0 \times 10^{-6}$	20	2
		28.5	$1.0 \times 10^{-5} \pm 1.6 \times 10^{-6}$	19	2
		34.5	$1.6 \times 10^{-5} \pm 1.4 \times 10^{-6}$	12	2
0.006	5.7	26.8	$9.1 \times 10^{-6} \pm 1.3 \times 10^{-6}$	21	2
		28.5	$9.9 \times 10^{-6} \pm 6.1 \times 10^{-7}$	19	2
		34.5	$1.2 \times 10^{-5} \pm 1.3 \times 10^{-6}$	16	2
0.01	8.5	27.0	$7.3 \times 10^{-6} \pm 8.9 \times 10^{-7}$	26	3
		28.0	$8.5 \times 10^{-6} \pm 1.1 \times 10^{-7}$	23	2
0.01	5.7	26.3	$5.7 \times 10^{-6} \pm 4.2 \times 10^{-7}$	34	2
		28.0	$6.0 \times 10^{-6} \pm 7.5 \times 10^{-7}$	32	2
0.014	8.5	27.3	$5.9 \times 10^{-6} \pm 3.1 \times 10^{-7}$	33	2
		28.5	$7.1 \times 10^{-6} \pm 5.1 \times 10^{-7}$	27	2

[a] pH 5.7 with acetic acid/acetate buffer, pH 8.5 with boric acid/borate buffer. [b] SD, standard deviation.

hydrolysis rate increases rapidly with increasing temperatures. Generally, the terbufos hydrolysis rate at pH 8.5 increases by ~100 and 70% for a 7 °C increase in temperature in 0.003 and 0.006 M buffers, respectively, and it increases by 85 and 30% at pH 5.7. Finally, the effect of temperature is more important at basic pH values, and the effect of temperature is more important than that of buffer concentration in most natural water systems, where ionic strength does not vary as dramatically as temperature does.

Product Analysis. The Nash method confirmed the formation of HCHO during terbufos hydrolysis at pH 5.7, 8.5, and 10.75. The HCHO profiles from several terbufos hydrolysis experiments at 29 and 37 °C are shown in Figure 3. The formation of HCHO is slightly higher at higher temperatures. Furthermore, the more alkaline the reaction conditions become, the lower the HCHO formation is. It can be simply explained by the fact that HCHO can be oxidized by dissolved oxygen to formic acid more readily at alkaline pH values (*23*), because most experiments were not conducted under deoxygenated conditions. However, it is fortunate for humans that aerobic microorganisms in most natural aquatic environments can utilize low concentrations of HCHO (i.e., up to 1500 mg/L or 0.05 M) as food and convert it to CO_2 and water (*24*). However, to prove the above hypothesis experimentally, HCHO formation was monitored from a series of deoxygenated terbufos hydrolysis experiments at both pH 8.5 and 5.7. It was found that HCHO is produced at nearly equimolar concentrations of terbufos (i.e., at >90% of the starting 17 μM concentration of terbufos, see Figure 4 for a temporal HCHO profile of one such experiment). The results shown in Figures 3 and 4 are similar to the phorate hydrolysis (*20*), in which HCHO-producing hydrolysis pathways are responsible for the majority of phorate hydrolysis at pH 8.5.

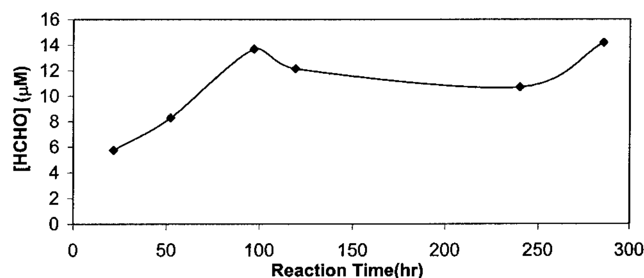

Figure 4. Formaldehyde production during deoxygenated terbufos hydrolysis at 28.5 °C, pH 8.5, and 0.01 M buffer concentration.

Table 4. Di-*tert*-butyl Disulfide Formation from 17 μM Terbufos Hydrolysis

exptl conditions	reaction time (days)	[di-*tert*-butyl disulfide] (μM)
0.014 M of boric acid/borate buffer and pH 8.5	4.0	5.5
	5.8	6.3
	6.9	10.2
	7.8	8.2
0.01 M of acetic acid/acetate buffer and pH 5.7	3.4	ND[a]
	4.9	ND
	5.2	6.0
	6.3	7.1
	7.2	7.3
	9.0	9.1
0.003 M of boric acid/borate buffer and pH 8.5	4.0	4.9
	5.8	5.4
	6.9	8.0
	7.8	7.3
0.003 M of acetic acid/acetate buffer and pH 5.7	4.0	ND
	4.9	ND
	5.8	5.7
	6.9	6.5
	7.8	6.7

[a] ND, not detectable.

A search of the mass spectral library NBS54K (NIST, Gaithersburg, MD) suggested that di-*tert*-butyl disulfide was one of the main products at pH 8.5 and 10.75 for all buffer concentrations tested, and it was also produced in terbufos hydrolysis experiments at pH 5.7. When pure di-*tert*-butyl disulfide was analyzed by GC-MS, it yielded the same retention time and the same mass spectrum. When the reaction mixture from a pH 8.5 hydrolysis experiment was subjected to methylation by diazomethane, phosphorodithioate, *O,O*-diethyl, *S*-methyl ester was tentatively identified by GC-MS analysis; its precursor was not found without methylation. When neat *O,O*-diethyl dithiophosphate was methylated by diazomethane, the same aforementioned compound was identified by GC-MS at the same retention time. Thus, it is confirmed that terbufos hydrolysis at pH 8.5 produces *O,O*-diethyl dithiophosphate (Figure 1d). However, *O,O*-diethyl dithiophosphate was not detected from terbufos hydrolysis at pH 5.7. This is especially intriguing when one considers that both HCHO and di-*tert*-butyl disulfide were still detected at pH 5.7. Can the P-containing hydrolysis product be *O,O*-diethyl phosphorothionic acid (Figure 1e) as the result of a P−S bond cleavage? Wolfe et al. (*25*) reported the formation of *O,O*-dimethyl phosphorothionic acid (Figure 1f) under acidic conditions from malathion hydrolysis (Figure 1g). However, the hydrolysis conditions for the malathion study were pH 2.5 and 87 °C; not only will the S atom be protonated significantly at such acidic pH (leading to a better leaving group), but the high temperature will also provide abundant kinetic energy for the P−S bond rupture pathway. If either *O,O*-diethyl dithiophosphate or *O,O*-diethyl phosphorothionic acid was produced during terbufos hydrolysis at pH 5.7, both should be identifiable via methylation. Lai et al. (*26*) did report the hydrolytic cleavage of a P−S bond in organophosphorus pesticides at 37 °C, but organophosphorus hydrolase was used as the catalyst and the hydrolysis of the only dithioate (i.e., the subgroup of organophosphorus pesticides that terbufos also belongs to), malathion, was the slowest.

A quantitative analysis of di-*tert*-butyl disulfide production was performed using the GC in the S mode with the same GC conditions as in the P mode, and the acquired data are presented in Table 4 and Figures 5 and 6. It is noteworthy that di-*tert*-butyl disulfide was not detected until after 3 days, although samples were collected and analyzed by GC from the very beginning. The production of di-*tert*-butyl disulfide was found to be higher at pH 8.5 than at pH 5.7, which is consistent with the known chemistry of thiols. Thiols are readily oxidized by oxidizers, such as O_2 and H_2O_2 (*27*, *28*). Furthermore, the oxidation of thiols has been found to be fastest at alkaline pH values (*29*, *30*). Under alkaline conditions, many thiols ionize, and the resulting ionized thiols oxidize more quickly to form disulfides (*29*). No measurement for *tert*-butyl thiol concentrations was attempted, but it is known that *tert*-butyl thiol is one of the least vulnerable toward oxidation among the alkanethiols (*31*). The temporal profile of di-*tert*-butyl disulfide under deoxygenated conditions (Figure 6) also supports this hypothesis, as the observed yield of the disulfide is somewhat higher and its formation is faster under oxygenated conditions (Figure 5) as compared to deoxygenated conditions (Figure 6).

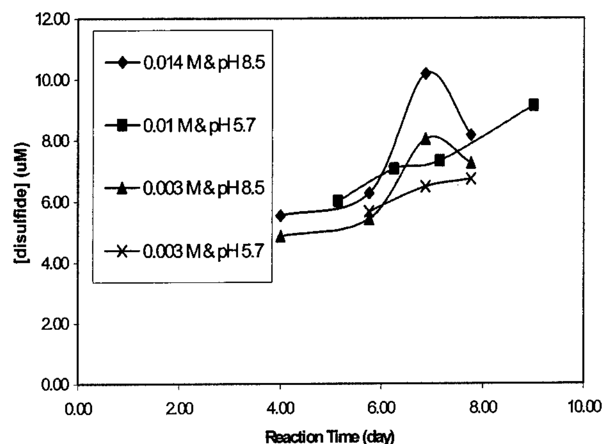

Figure 5. Time-dependent di-*tert*-butyl disulfide formation during terbufos hydrolysis.

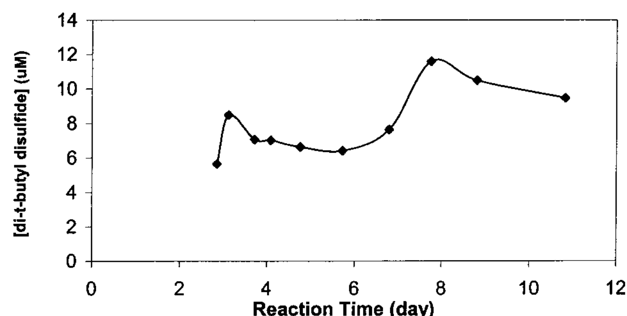

Figure 6. Di-*tert*-butyl disulfide production during deoxygenated terbufos hydrolysis in 0.01 M boric acid/borate buffer at pH 8.5 and 28.5 °C.

No H_2S was detected, which can be attributed to several factors. First, H_2S might not have been produced during the hydrolysis of terbufos. This would be surprising considering the structural similarities between phorate and terbufos (Figure 1). It is conceivable, however, because *O,O*-diethyl dithiophosphate (Figure 1d) seems to be rather stable in aqueous solutions (*32*). If the initial nucleophilic attack occurs mainly at the methylene carbon of the side chain instead of the phosphorus atom, then little H_2S would be produced (Figure 7). Second, the H_2S concentration may be below the detection limit of 10^{-7} M. This is possible, because the initial terbufos concentration of 17 μM is lower (due to solubility limitations) than the initial phorate con-

$$(EtO)_2P(=S)\text{–S–}CH_2\text{–S–t-Bu} \xrightleftharpoons{OH^- \text{ or } H_2O} (EtO)_2P(=S)\text{–}S^- + HO\text{–}CH_2\text{–S–t-Bu}$$

$$HO\text{–}CH_2\text{–S–t-Bu} \xrightarrow{OH^-} \text{t-Bu–SH} + HCHO$$

$$2\ \text{t-Bu–SH} + 1/2\ O_2 \rightleftharpoons \text{t-Bu–S–S–t-Bu} + H_2O$$

Figure 7. Proposed mechanism for terbufos hydrolysis.

Table 5. Data of Microtox Toxicity Assay

compound	EC_{50} (mg/L)	EC_{50} (mM)
phenol	22	0.23
terbufos	too great[a]	too great[a]
P–SH[b]	2.1	1.2×10^{-2}
di-*tert*-butyl disulfide	0.24	1.3×10^{-3}

[a] Larger than the initial concentration of 17 μM. [b] *O,O*-Diethyl dithiophosphate.

Table 6. Time Profile of Toxicity during 17 μM Terbufos Hydrolysis at 0.01 M Buffer

	EC_{50} (mg/L)	
reaction time (days)	pH 8.5 with boric acid/borate buffer	pH 5.7 with acetic acid/acetate buffer
0	66	37
3.3	45	51
7.3	53	89
10.3	120	68

centration of 69 μM, and only low concentrations of H_2S were detected during phorate hydrolysis (*20*). Finally, it is known that HCHO can significantly accelerate the oxidation of H_2S under alkaline conditions (*33*).

Both HCHO and di-*tert*-butyl disulfide were expected products of terbufos hydrolysis due to the structural similarity of terbufos and phorate, and they are indeed observed. On the basis of the results from product analysis, a reaction mechanism is proposed for the hydrolysis of terbufos (Figure 7). This mechanism may fit the pH 8.5 case better because *O,O*-diethyl dithiophosphate was not detected at pH 5.7.

Toxicity Analysis. Microtox, a simple and economical alternative to acute lethality tests, such as *Daphnia* or rainbow trout bioassay (*34*), has been widely used (*35–37*). The U.S. EPA is considering adopting Microtox as a quick screening test for toxicity. The test parameter EC_{50} is the concentration of a compound (or the dilution of a sample) that reduces the luciferase activity of *Photobacterium phosphoreum* by 50%. EC_{50} values for a phenol standard, terbufos, di-*tert*-butyl disulfide, and *O,O*-diethyl dithiophosphate are shown in Table 5. Terbufos was not found to be toxic by Microtox at the concentration used in this study (the maximum concentration of terbufos is only 17 μM due to aqueous solubility limitations), but terbufos was shown to be about as toxic as phorate toward the northern bobwhite (*38*). Di-*tert*-butyl disulfide was found to be >100 times as toxic as phenol on a molar basis and ~10 times as toxic as diethyl disulfide, a hydrolysis product of phorate (*20*)! The higher toxicity of di-*tert*-butyl disulfide is probably due to the higher hydrophobicity of the *tert*-butyl group (making it easier to penetrate the lipid bilayer of the cell membrane) than the ethyl group in diethyl disulfide. Because the redox chemistry surrounding the disulfide bond is critical in biochemistry, the presence of disulfides can potentially disrupt the cellular metabolism (*39*). Hence, similar to the case of phorate (*20*), one of the hydrolysis products of terbufos, a di-*tert*-butyl disulfide, was again found to be very toxic.

The relatively high toxicity of *O,O*-diethyl dithiophosphate is unfortunately less conclusive, because the identities of impurities in the technical product (~90% pure) are unavailable from the manufacturer, Aldrich Chemical Co. In addition, the significant formation of HCHO, a suspected carcinogen (*40*), from terbufos hydrolysis (Figures 3 and 4) is also noteworthy.

The toxicity of the hydrolysis reaction mixtures was also monitored throughout the experiments to confirm the proposed hydrolysis mechanism. The results agree well with the hydrolysis results, the product identification, and individual compound toxicity (Table 5), because the toxicity increased for the first few days as di-*tert*-butyl disulfide, which is more toxic than terbufos itself, was gradually produced. Toxicity decreased again (Table 6) as di-*tert*-butyl disulfide was subsequently degraded to some other less toxic compounds. At pH 5.7, the toxicity decreased during the initial period of terbufos hydrolysis. This is consistent with the fact that less di-*tert*-butyl disulfide has been detected during terbufos hydrolysis at pH 5.7 (Figure 5). After the initial decrease, some toxic hydrolysis products were formed and hence the toxicity increased. The toxicity time profiles are different for the two pH scenarios (Table 6) because the terbufos hydrolysis rate and product formation are pH-dependent (Table 1).

The quantitative analyses of di-*tert*-butyl disulfide formation also agree well with the toxicity profile. As can be seen in Figure 5 and Table 4, di-*tert*-butyl disulfide was produced gradually, so that the toxicity increased with time (Table 6). Terbufos then further hydrolyzed to other less toxic compounds accompanied by decreasing toxicity. Because terbufos hydrolysis is base-catalyzed, the reaction progressed more quickly and the di-*tert*-butyl disulfide reached a maximum concentration at an earlier stage at pH 8.5 than at pH 5.7 (Figure 5). At pH 8.5, this resulted in an increase in toxicity from the beginning of the experiments to a point. A subsequent decrease in toxicity was observed, as the disulfide was further degraded.

Conclusions. The observed hydrolysis rate constants of terbufos are similar to those of phorate, although the *tert*-butyl group of terbufos eliminates an intramolecular nucleophilic attack (the ethyl group in phorate would not be a steric obstacle). The calculated hydrolysis rate constants for acid-catalyzed, base-catalyzed, and neutral pathways are similar to those of other organophosphorus pesticides (*8*, *9*, *11*, *41*, *42*). Hydrolysis of terbufos results in a very toxic product, a di-*tert*-butyl disulfide (formed by hydrolysis of terbufos followed by oxidative coupling of *tert*-butyl thiol molecules), and one possibly toxic product, *O,O*-diethyl dithiophosphate (detected only under alkaline conditions). Formaldehyde, a suspected carcinogen, was also produced, although the soil microorganisms can utilize low concentrations of HCHO. It is difficult to predict how important these products are under ambient conditions, when dilution and oxidation also affect the environmental fate of terbufos. Particularly, the oxidative coupling of *tert*-butyl thiol to yield di-*tert*-butyl disulfide may be a strong function of ambient concentrations of terbufos and its hydrolysis product, *tert*-butyl thiol. Although very sophisticated protection equipment is available for human handlers of these organophosphorus compounds, the exposure to them and their hydrolysis products can still cause considerable harm to aquatic organisms. Degradation products of terbufos and phorate and their toxicity should be considered when their ecological impact is assessed.

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LITERATURE CITED

(1) Khan, S. U., Ed. *Pesticides in the Soil Environment*; Elsevier: Amsterdam, The Netherlands, 1980; pp 23−47.

(2) Howard, P. H. *Handbook of Environmental Fate and Exposure Data for Organic Chemicals*; Lewis Publishers: Chelsea, MI, 1991; pp 34−89.

(3) Hudson, R. H., Tucker, R. K., Haegele, M. A., Eds. *Handbook of Toxicity of Pesticides to Wildlife*; U.S. Department of the Interior, Fish and Wildlife Service: Washington, DC, 1984; pp 34−67.

(4) Thurston, R. V.; Gilfoil, T. A.; Meyn, E. L.; Zajdel, R. K.; Aoki, T. I.; Veith, G. D. Comparative toxicity of ten organic chemicals to ten common aquatic species. *Water Res.* **1985**, *19*, 1145−1155.

(5) Smith, G. J. *Toxicology and Pesticide Use in Relation to Wildlife: Organophosphorus and Carbamate Compounds*; Smoley, C. K., Ed.; Plenum Press: New York, 1993; pp 123−145.

(6) Juarez, S. L. M.; Sanchez, J. Toxicity of the organophosphorus insecticide Metamidophos (*O,S*-dimethyl phosphramidothioate) to larvae of the freshwater prawn *Macrobrachium rosenbergii* (De Man) and the blue shrimp *Penaeus stylirostris*. *Bull. Environ. Contam. Toxicol.* **1989**, *43*, 302−308.

(7) Fernandez-Casalderrey, A.; Ferrando, M. D.; Andreu-Moliner, E. Endosulfan and diazinon toxicity to the freshwater rotifier *Brachionus calyciflorus*. *J. Environ. Sci. Health*−*Pestic. Food Agric. Wastes* **1992**, *B27*, 155−164.

(8) Wanner, O.; Egli, T.; Fleishmann, T.; Lanz, K.; Reichert, P.; Schwarzenbach, R. P. The behavior of the insecticides disulfoton and thiometon in the Rhine river? A chemodynamic study. *Environ. Sci. Technol.* **1989**, *23*, 1232−1242.

(9) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*; Wiley: New York, 1993; pp 393−399.

(10) Zhang, Q.; Pehkonen, S. O. Oxidation of diazinon by aqueous chlorine: kinetics, mechanisms and product studies. *J. Agric. Food Chem.* **1999**, *47*, 1760−1766.

(11) Faust, S. D.; Gomaa, H. M. Chemical hydrolysis of some organic phosphorus and carbamate pesticides in aquatic environments. *Environ. Lett.* **1972**, *3*, 171−201.

(12) National Agricultural Statistics Service. *Agricultural Chemical Usage: 1996 Restricted Use Pesticides Summary*; Washington, DC, 1997.

(13) Chapman, R. A.; Harris, C. R. Insecticidal activity and persistence of terbufos, terbufos sulfoxide and terbufos sulfone in soil. *J. Econ. Entomol.* **1980**, *73*, 536−543.

(14) Szeto, S. Y.; Brown, M. J.; Mackenzie, J. R.; Vernon, R. S. Degradation of terbufos in the soil and its translocation into cole crops. *J. Agric. Food Chem.* **1986**, *34*, 876−879.

(15) Ware, G. W. *Fundamentals of Pesticides*; Thomson: Fresno, CA, 1986; pp 34−67.

(16) Bowman, B. T.; Sans, W. W. Adsorption, desorption, soil mobility, aqueous persistence and octanol−water partitioning coefficients of terbufos, terbufos sulfoxide and terbufos sulfone. *J. Environ. Sci. Health* **1982**, *B17*, 447−462.

(17) Chapman, R. A.; Cole, C. M. Observations on the influence of water and soil pH on the persistence of insecticides. *J. Environ. Sci. Health* **1982**, *B17*, 487−504.

(18) Howe, G. E.; Marking, L. L.; Bills, T. D.; Rach, J. J.; Mayer, F. L. Effects of water temperature and pH on toxicity of terbufos, trichlorfon, 4-nitrophenol and 2,4-dinitrophenol to the amphipod *Gammarus pseudolimnaeus* and rainbow trout (*Onchorhynchus mykiss*). *Environ. Toxicol. Chem.* **1994**, *13*, 51−66.

(19) Wolfe, M. F.; Kendall, R. J. Age-dependent toxicity of diazinon and terbufos in European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*). *Environ. Toxicol. Chem.* **1998**, *17*, 1300−1312.

(20) Hong, F.; Pehkonen, S. O. Hydrolysis of phorate using simulated environmental conditions: rates, mechanisms, and product analysis. *J. Agric. Food Chem*. **1998**, *46*, 1192−1199.

(21) Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsh reaction. *Biochem. J.* **1953**, *55*, 416−421.

(22) Smith, R. V.; Erhardt, P. W. Nash determination for formaldehyde in the presence of bisulfite. *Anal. Chem.* **1975**, *47*, 2462−2464.

(23) Budavari, S., O'Neil, M. J., Smith, A., Heckelman, P. E., Eds. *The Merck Index*, 11th ed.; Merck: Rahway, NJ, 1989.

(24) Sawyer, C. N.; McCarty, P. L.; Parkin, G. F. *Chemistry for Environmental Engineering*, 4th ed.; McGraw-Hill: New York, 1994; p 345.

(25) Wolfe, N. L.; Zepp, R. G.; Gordon, J. A.; Baughman, G. L.; Cline, D. M. Kinetics of chemical degradation of malathion in water. *Environ. Sci. Technol.* **1977**, *11*, 88−93.

(26) Lai, K.; Stolowich, N. J.; Wild, J. R. Characterization of P−S bond hydrolysis in organophosphorothioate pesticides by organophosphorus hydrolase. *Arch. Biochem. Biophys.* **1995**, *318*, 59−64.

(27) Kice, J. L.; Marvell, E. N. *Modern Principles of Organic Chemistry*, 2nd ed.; Macmillan: New York, 1974; pp 78−99.
(28) Fessenden, R. J.; Fessenden, J. S. *Fundamentals of Organic Chemistry*; Harper Collins: New York, 1990; pp 123−135.
(29) Halliwell, B. Reactive oxygen species in living systems. *Am. J. Med.* **1991**, *14*, 91−99.
(30) Quijano, C.; Alvarez, B.; Gatti, R. M.; Augusto, O.; Radi, R. Pathways of peroxynitrite oxidation of thiol groups. *Biochem. J.* **1997**, *322*, 167−173.
(31) Cullis, C. F.; Hopton, J. D.; Trimm, D. L. Oxidation of thiols in gas−liquid systems. 1. Reaction in the absence of added metal catalysts. *J. Appl. Chem*. **1968**, *18*, 330−335.
(32) Hong, F.; Pehkonen, S. O.; Brooks, E. Pathways for the hydrolysis of phorate: product studies by ^{31}P-NMR and GC-MS. *J. Agric. Food Chem.* **2000**, *48*, 3013−3017.
(33) Chen, K. Y.; Morris, J. C. Kinetics of oxidation of aqueous sulfide by O_2. *Environ. Sci. Technol.* **1972**, *6*, 529−537.
(34) Munkittrick, K. R.; Power, E. A. The relative sensitivity of Microtox, daphnid, rainbow trout, and fathead minnow acute lethality tests. *Environ. Toxicol. Water Qual.* **1991**, *6*, 35−62.
(35) Indorato, A. M.; Snyder, K. B.; Usinowics, P. J. Toxicity screening using Microtox analyzer. In *Toxicity Screening Procedures Using Bacterial Systems*; Liu, D., Dutka, P. J., Eds.; Dekker: New York, 1984; pp 37−54.
(36) Ghosh, S. K.; Doctor, P. B.; Bhatnagar, V. K.; Yadav, S.; Derasari, A.; Kulkarni, P. K.; Kashyap, S. K. Response of three microbial test systems to pesticides. *Bull. Environ. Contam. Toxicol.* **1997**, *58*, 482−488.
(37) Ruiz, M. J.; López-Jaramillo, L.; Redondo, M. J.; Font, G. Toxicity assessment of pesticides using the Microtox test: application to environmental samples. *Bull. Environ. Contam. Toxicol.* **1997**, *59*, 619−625.
(38) Hill, E. F.; Camardese, M. B. Toxicity of anticholinesterase insecticides to birds: technical grade versus granular formulations. *Ecotoxicol. Environ. Saf.* **1984**, *8*, 551−563.
(39) Mathews, C. K., van Holde, K. E., Eds. *Biochemistry*; Cumings: Redwood City, CA, 1990; pp 234−245.
(40) Perara, F.; Petito, C. Formaldehyde: a question of cancer policy? *Science* **1982**, *216*, 1285−1291.
(41) Mabey, W.; Mill, T. Critical review of hydrolysis of organic compounds in water under environmental conditions. *J. Phys. Ref. Data* **1978**, *7*, 383−415.
(42) Dannenberg, A.; Pehkonen, S. O. Investigation of the heterogeneously catalyzed hydrolysis of organophosphorus pesticides. *J. Agric. Food Chem.* **1998**, *46*, 325−334.

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