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Phototransformation of Carboxin in Water. Toxicity of the Pesticide and Its Sulfoxide to Aquatic Organisms

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Sunlight exposure of aqueous suspensions of carboxin (**1**) causes its phototransformation to sulfoxide **2** and minor components. Similar effects are observed in the presence of humic acid or nitrate or at different pH values. Photoproducts **2**−**9** were isolated by chromatographic techniques and/or identified by spectroscopic means. Carboxin **1** and its main photoproduct sulfoxide **2** were tested to evaluate acute toxicity to primary consumers typical of the aquatic environment: the rotifer *Brachionus calyciflorus* and two crustaceans, *Daphnia magna* and *Thamnocephalus platyurus*. Chronic tests comprised a producer, the alga *Pseudokirchneriella subcapitata*, and a consumer, the crustacean *Ceriodaphnia dubia*.

KEYWORDS: Carboxin; solar light; nitrate; humic acid; toxicity; *Brachionus calyciflorus*; *Daphnia magna*; *Thamnocephalus platyurus*; *Pseudokirchneriella subcapitata*; *Ceriodaphnia dubia*

INTRODUCTION

Pesticides are largely used to ensure crop yields and high quality, and the variety and quantities of these substances present in surface waters have dramatically increased. Most pesticides resistant to chemical and/or photochemical degradation under typical environmental conditions are detected in food and water (*1*). In recent years, many studies have reported the possible adverse effects that the presence of these pesticides may have for human health and the equilibrium of ecosystems (*2*−*5*). However, pesticides may be subjected to physical, chemical, and biological transformations in the environment. Therefore, the presence and possible effects of the transformation products should be investigated, too. Here we report the photolysis of the pesticide carboxin (**1**) in water by sunlight exposure. Carboxin is a systemic fungicide used to control plant diseases caused by *Basidiomycetes* (*6*). Recent studies have described the photochemical behavior of the pesticide when irradiated with UV light (filter Pyrex) in organic solvent (*7*) or in the presence of humic substances and soil (*8*) and with a halogen lamp in the presence of sensitizers (*9*). In this study we examine the irradiation in water with solar light as close to natural conditions as possible. The effect of pH or commercially available humic acid or nitrate on the rate of degradation is also discussed. Humate and nitrate, which are likely to be encountered in aquatic environments, are reported to be capable of inducing the photodegradation of organic compounds. The former have been shown to sensitize oxygenation and other reactions involving electronic energy transfer (*10*), whereas nitrate promotes essentially OH radical formation (*11*).

Carboxin, continuously discharged in the environment, mainly on the soil, can reach surface waters due to phenomena of runoff. Therefore, the present study evaluates the ecotoxicological effects of the pesticide and its main photoproducts on nontarget aquatic organisms.

MATERIALS AND METHODS

Chemicals. Carboxin, analytical standard grade (99%), was supplied by Labservice Analytika S.r.l.; all other chemicals were obtained from Aldrich.

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H and at 125 MHz for ^{13}C on a Varian 500 Fourier transform NMR spectrometer. Electronic impact mass spectra (EI-MS) were obtained with an HP 6890 spectrometer equipped with an MS 5973 N detector. UV−vis spectra were recorded in methanol on a Perkin-Elmer Lambda 7 spectrophotometer. IR spectra were recorded on a Jasco FT/IR-430 instrument. The HPLC apparatus consisted of an Agilent 1100 HPLC system equipped with a refractive index detector. The column was a Phenomenex Hydro RP-18, 4 μm, 250 × 4.5 mm. Analytical TLC was made on Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness (Merck). Preparative TLC was performed on Kieselgel 60 F_{254} plates with 0.5 or 1 mm film thickness (Merck). Flash column chromatography (FCC) was conducted on Kieselgel 60, 230−400 mesh (Merck), at medium pressure.

Phototransformation of Carboxin by Natural Solar Light. A suspension of carboxin (20 mg) in 300 mL of deionized water was exposed to natural sunlight in Pyrex flask, under aerobic conditions, at Naples (Italy) in October 2003 (temperature range of 20−35 °C).

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Table 1. Product Distribution by Sunlight Irradiation of Carboxin in Water after 4 Days

reaction condition[a]	product distribution[b] (%)							
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**
pH 7	55	23	5	14	1	2		
pH 10	76	20	3			1		
pH 2	47	38	7	2	<1	-	5	2
humic acid, 10%	63	24	3	10	<1	<1	<1	
KNO_3, 5%	27	45	5	18	5	<1	<1	

[a] Suspension of carboxin (20 mg) in 300 mL of water after saturating with oxygen. [b] Percentages have been deduced by ^{1}H NMR of the mixture extracted with ethyl acetate.

Similar experiments were carried out by adjusting the pH of suspension to 2 with 10^{-3} M HCl and to 10 with 10^{-4} M KOH, in the presence of KNO_3 (10 mg/L), with humic acid (5 mg/L). Each experiment was performed in duplicate, with one set of dark controls. After 4 days of sunlight exposure, each reaction mixture was extracted with ethyl acetate, yielding 10−15 mg of crude mixture. The organic layer was analyzed by ^{1}H NMR, and product distribution was reported for each experiment in **Table 1**. The mixture was chromatographed by reverse phase C-18 HPLC [$H_2O/CH_3OH/CH_3CN$ (5:3:2)] using a refractive index detector to give unreacted carboxin (30−55%) and the photoproducts (altogether 20−35%). The aqueous layer was analyzed by ^{1}H NMR and showed the presence of only one product, which was identified as oxanilic acid (**7**). All of the products were fully characterized by spectral means. Compounds **2**, **3**, **8**, and **9** were identified by comparing the spectroscopic data with those reported in the literature (*9*, *12*, *13*). Compound **5** was identified by comparison of spectral data with those of a pure sample suitably prepared. The unreported spectral data for products **3** (*12*), **4** (*14*), **5** (*15*), **6** (*7*), and **7** (*8*) are given below.

Ketoamide 3: ^{13}C NMR ($CDCl_3$) δ 25.3 (Me), 33.8 (CH_2S), 71.8 (CH_2O), 92.1 (C-2), 119.8 (C-2′), 125.5 (C-4′), 129.2 (C-3′), 136.1 (C-1′), 156.2 (CON), 189.7 (CO); MS, *m*/*z* 251 $[M]^+$, 103.

Acetate 4: IR ($CHCl_3$) 2852, 1717 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.08 (s, 3 H, −Me), 2.93 (t, J = 6.3 Hz, 2H, CH_2S), 4.33 (t, J = 6.3 Hz, 2H, CH_2O); ^{13}C NMR ($CDCl_3$) δ 23.7 (Me), 38.7 (CH_2S), 68.1 (CH_2O), 172.6 (CO_2); MS, *m*/*z* 87 $[M - SH]^+$, 60, 43.

Disulfide 5: ^{1}H NMR ($CDCl_3$) δ 2.88 (t, J = 6.3 Hz, 4H, $2CH_2S$), 2.98 (br s, 2H, 2OH), 3.89 (t, J = 6.3 Hz, 4H, $2CH_2O$); ^{13}C NMR ($CDCl_3$) δ 41.2 (CH_2S), 60.3 (CH_2O); MS, *m*/*z* 154 $[M]^+$, 92, 79, 64, 45.

3-[(2-Hydroxyethyl)thio]-4-methylquinolin-2(1H)-one (6): mp 153−154 °C (CH_2Cl_2/hexane); IR ($CHCl_3$) 3389, 1641 cm^{-1}; NMR ($CDCl_3$) δ 2.92 (s, 3H, Me), 3.04 (t, J = 5.2 Hz, 2H, CH_2S), 3.70 (t, J = 5.2 Hz, 2H, CH_2O), 4.35 (br s, 1H, OH), 7.30 (t, J = 7.5 Hz, 1H, H-6), 7.45 (d, J = 7.5 Hz, 1H, H-8), 7.58 (t, J = 7.5 Hz, 1H, H-7), 7.78 (d, J = 7.5 Hz, 1H, H-5), 11.75 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 18.2 (Me), 38.8 (CH_2S), 60.2 (CH_2O), 116.7 (C-8), 120.6 (C-10), 123.2 (C-6), 124.4 (C-3), 125.5 (C-5), 131.5 (C-7), 137.2 (C-9), 155.7 (C-4), 161.1 (C-2); MS, *m*/*z* 235 $[M]^+$, 204, 143, 77, 43.

Oxanilic Acid (7): IR (KBr, wafer) 3473, 1697 cm^{-1}; ^{1}H NMR (DMSO) δ 7.03 (t, J = 7.3 Hz, 1H), 7.27 (t, J = 7.3 Hz, 2H), 7.75 (d, J = 7.3 Hz, 2H), 10.18 (br s, 1 H); ^{13}C NMR (DMSO) δ 119.8 (C-2′), 125.5 (C-4′), 129.2 (C-3′), 136.1 (C-1′), 163.3 (CON), 165.5 (COOH); MS, *m*/*z* 167 $[M]^+$, 148 $[M - OH]^+$.

Enol 9: ^{13}C NMR ($CDCl_3$) δ 21.3 (Me), 39.1 (CH_2S), 59.7 (CH_2O), 93.1 (C-3), 120.3 (C-2′), 124.5 (C-4′), 129.0 (C-3′), 137.3 (C-1′), 171.1 (C-2), 183.1 (C-7); MS, *m*/*z* 253 $[M]^+$, 193, 135.

Sunlight Irradiation of Sulfoxide 2 in Water for 1 Month. Twenty milligrams of this compound was dissolved in deionized water and exposed to solar light for 1 month (between October 4 and November 3, 2003). After extraction with ethyl acetate, ^{1}H NMR analysis of the organic layer showed the presence of only the starting sulfoxide **2**.

Preparation of Products 4, 5, and 7. Compound **8** (25 mg), synthesized as reported in the literature (*9*), was dispersed in deionized water (250 mL) and exposed to natural sunlight for 4 days. Then, the mixture was extracted with ethyl acetate, giving acetate **4** and compound **5** (in ∼2:1 molar ratio) in the organic layer, whereas the aqueous extract, after evaporation, gave the oxanilic acid **7**.

Preparation of Disulfide 5. A solution of 1-mercaptoethanol (30 mg) in deionized water (300 mL) was saturated with oxygen and exposed to solar light for 4 days. Then, the solution was extracted with ethyl acetate, and the organic layer, analyzed by ^{1}H NMR, showed the presence of a product whose signals matched exactly those of compound **5**.

Toxicity Testing. Acute toxicity tests were performed on primary consumers typical of the aquatic environment: the rotifer *Brachionus calyciflorus* and two crustaceans, *Daphnia magna* and *Thamnocephalus platyurus*. Chronic tests (sublethal endpoints) comprised a producer, the alga *Pseudokirchneriella subcapitata*, and a consumer, the crustacean *Ceriodaphnia dubia*.

Carboxin and its photoderivative were previously dissolved in dimethyl sulfoxide (Carlo Erba, Milan, Italy), and, in the final test solutions, DMSO concentration was kept constant at 0.01% (v/v). A solvent-only control was included in each experiment to detect the possible effect of the vehicle. For all of the bioassays dissolved oxygen and pH were measured in each sample both at the start and at the end of testing. At the same time as acute and chronic toxicity tests, reference assays were performed with potassium dichromate (Aldrich Chemical, St. Louis, MO) for all of the organisms except *C. dubia*, for which pentahydrate copper sulfate (Aldrich) was used.

Acute Bioassays. Juveniles (age, 0−2 h) of the rotifer *B. calyciflorus* were hatched from cysts provided by MicroBioTest, Nazareth, Belgium, after 16−18 h of incubation under a light source of 3000−4000 lx at 25 °C in synthetic reconstituted medium (moderately hard medium EPA-600/4-85-013) and then exposed to the test sample (*16*). Five test dilutions were prepared in a 50% dilution series for each sample with six replicates of five animals (0.3 mL of test solution, slightly different from the ASTM procedure applied). Test duration was 24 h, temperature was 25 °C, in the dark. The test parameter considered was mortality, and the concentration found to kill 50% of the rotifers in 24 h was indicated as the LC_{50} (*16*).

The bioassay on the anostracan crustacean *T. platyurus* was conducted using second- and third-instar fairy shrimp larvae hatched from cysts provided by MicroBioTest, after 20−22 h of incubation at 25 °C in synthetic reconstituted freshwater (the same moderately hard EPA medium as rotifers) under continuous illumination (light source of 3000−4000 lx). Tests were performed in 24-well plates, 10 crustaceans per well (1.0 mL of test solution), 3 replicates per concentration, 5 concentrations, and a negative control. Test duration was 24 h, temperature was 25 °C, in the dark. The test parameter considered was mortality, and the concentration found to kill 50% of the crustaceans in 24 h was indicated as the LC_{50}.

The test on *D. magna* Straus, during 24 h of exposure, was performed on young organisms obtained from our laboratory cultures, <24 h old, at 20 °C in the dark, following International Standard Organization procedure 6341 for acute toxicity tests (*17*). The synthetic reconstituted freshwater, aerated before use, was the ISO hard medium (hardness of 250 mg/L expressed as $CaCO_3$). Tests were performed with five daphnids per vessel (10 mL of test solution), four replicates for each of the five concentrations. The test endpoint was the inhibition of mobility, and the concentration found to immobilize 50% of the crustaceans in 24 h was indicated as the EC_{50}.

Chronic Bioassays. The algal growth inhibition test was run in 72 h according to International Standard Organization procedure 8692 (*18*). The algal inoculum was taken from an exponentially growing preculture and added to 25 mL of test solution to obtain an initial cell density of the order of 10^4 cells/mL. Each compound was tested in five concentrations, three replicates, and a negative control. Flasks were placed in a growth chamber at 25 °C under continuous illumination (8000 lx). The cell density was measured at 0 time and every 24 h for 3 days by an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary, Instrumentation Laboratory, Miami, FL), and from these data the algal growth inhibition in percentage was calculated by integrating the mean values from t_0 to t_{72} h (area under the curve).

The test on *C. dubia* was based on a population growth inhibition in 7 days and performed on young organisms, <24 h old, obtained by acyclical parthenogenesis of individual adult females for at least three

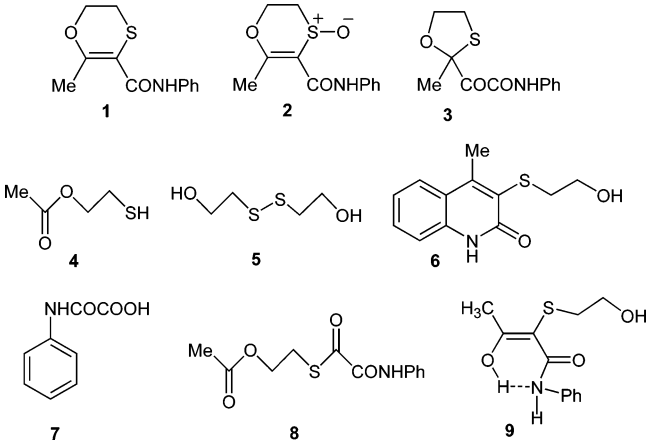

Figure 1. Carboxin and its photoproducts.

generations. The first females were born from the hatching of ephippia provided by MicroBioTests. Organisms were exposed individually in beakers with 20 mL of test solution for 7 days to seven concentrations (2-fold dilutions) in synthetic reconstituted aerated hard ISO medium (total hardness of 250 mg/L as $CaCO_3$). Ten replicates were incubated at 25 °C with a 16:8 h light/dark cycle (500 lx) (*19*). Daphnids were fed at each renewal with 100 μL of a suspension of the alga *P. subcapitata* (4×10^8 cells/mL), food fish (5 g/L), and yeast (5 g/L). Organisms were monitored for survival, and released neonates were counted each day prior to renewals and then discharged. The parameter considered was the population growth inhibition, so that from the comparison between the number of offspring born from live or dead mothers at the end of the test in the sample batch and the control it was possible to calculate the concentration that gave rise to 50% population growth inhibition, indicated as the EC_{50} (*19*).

Data Analysis. All results, except the algal test, were analyzed using the program Toxcalc (*20*). For acute toxicity tests, the LC_{50} and EC_{50} with 95% confidence intervals were calculated by concentration/response regression using probit or the trimmed Spearman−Karber method, as appropriate. For the test with *C. dubia*, the value of EC_{50} with 95% confidence intervals was calculated using the maximum likelihood−logit method. Inhibition (percentage) values of algae were reported against log-transformed data of concentrations (in micromolar) and processed by a regression analysis technique to obtain the respective IC_{50} value (the test concentration corresponding to 50% reduction in growth relative to the control).

RESULTS AND DISCUSSION

After 4 days of exposure to sunlight, the water suspension of carboxin **1** (2.8×10^{-4} M) was extracted with ethyl acetate and the organic layer was analyzed by ^{1}H NMR. No appreciable variations were observed using more dilute solutions ($\sim 10^{-5}$ M).

The separation of this mixture (12 mg) by chromatographic methods allowed us to isolate in addition to unreacted carboxin (50%) five compounds, which were identified as sulfoxide **2**, ketoamide **3**, acetate **4**, disulfide **5**, and quinolinone **6** (**Figure 1**) by spectroscopic means (^{1}H NMR, ^{13}C NMR, MS, IR) and/or by comparison of some spectroscopic data with those previously reported (*8*, *12*, *13*). A similar procedure was carried out by sunlight exposing aqueous suspensions of carboxin at pH 2 and 14, in the presence of humic acid (10%) or with nitrate (5%). The conversion percentage and the composition of each mixture was evaluated by the ^{1}H NMR spectrum of the organic layer and are reported in **Table 1**.

In acidic conditions the ester **8** and the enol **9** were identified (**Figure 1**). Under all of the conditions used, evaporation of the aqueous layer furnished a compound that was spectroscopically identified as oxanilic acid **7** (**Figure 1**). A control experiment showed that this compound is obtained together with acetate **4** and disulfide **5** when a suspension of ester **8** is exposed to sunlight in water. Disulfide **5** is the oxidation product of mercaptoethanol formed by hydrolysis of acetate **4**. In all of the experiments dark controls for the same length of time showed no change of carboxin.

As reported in **Table 1**, carboxin is readily photodecomposed by natural sunlight, giving mainly sulfoxide **2** and acetate **4**. Ketoamide **3** is also found, whereas disulfide **5**, quinolinone **6**, and ester **8** are obtained in very small amounts. Environmental effects such as pH variation or the presence of humate appear

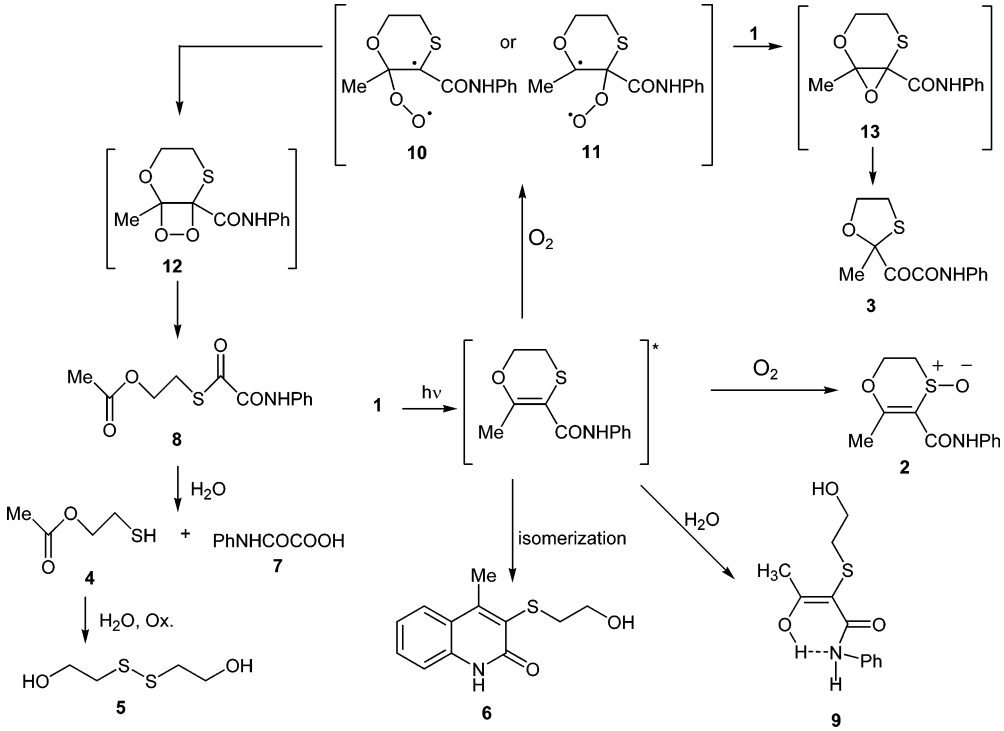

Figure 2. Mechanism of the photodegradation of carboxin.

Table 2. Toxicity Tests of Carboxin and Its Sulfoxide **2** toward Aquatic Organisms

	$L(E)C_{50}$ in mg/L for acute toxicity tests[a]			$L(E)C_{50}$ in mg/L for acute toxicity tests[a]	
compound	*B. calyciflorus*	*T. platyurus*	*D. magna*	*C. dubia*	*P. subcapitata*
carboxin **1**	5.0 (2.60–7.32)	61.00 (55.25–67.35)	22.59 (19.04–26.80)	0.64 (0.52–0.73)	2.41 (2.09–2.77)
sulfoxide **2**	4.10 (2.72–6.19)	56.57 (40.94–78.17)	NE[b] (80 ppm)	0.66 (0.31–0.79)	NE (30 ppm)

[a] Ninety-five percent confidence limits in parentheses. [b] No effect.

to have little influence on the photodegradation rate, whereas a significant increase is observed in the presence of the nitrate as expected on the basis of its property of inducing photo-oxidation (*11*).

Formation of the products can be explained on the basis of photo-oxidative transformations as the main light-induced pathways. According to previous studies (*7*), excited states of the pesticide, formed directly by the absorption of the solar radiation [carboxin exhibits an absorption band with a maximum at 292 nm (log ϵ of 3.2)], can react with ground-state oxygen, which adds to sulfur or to the double bond leading to sulfoxide **2** or to the radicals **10** or **11** (**Figure 2**). The latter afford ester **8** via the unstable dioxetane **12**, whereas intermolecular reactions should be involved in the formation of ketoamide **3** via the intermediate **13**. These products could be formed via other oxygenating species such as singlet oxygen generated by energy transfer by the excited **1** (*7*). Control experiments showed that the reaction is largely inhibited by DABCO (10^{-3} M) as a quencher of singlet oxygen or energy transfer reactions (20% of photodegradation after 4 days).

As proven by control experiments, hydrolysis of ester **8** leads to acetate **4** and acid **7**. Further decomposition of compound **4** gives disulfide **5**. Quinolinone **6** is a photoisomerization product and is formed also in the absence of oxygen (*7*). Enol **9** is formed by both acid- and light-induced addition of H_2O to carboxin. Indeed, this compound is not found under neutral and basic conditions, and carboxin was quantitatively recovered under acid conditions in the dark after 4 days.

Under all conditions used sulfoxide **2** is the main photoproduct (20−30%), and it is also highly photostable. Therefore, we examined the toxicity to aquatic organisms of sulfoxide as well as that of the parent compound.

Acute toxicity data, expressed as median effective concentrations (LC_{50} and EC_{50}) of carboxin and its sulfoxide, are reported in **Table 2**. The photoproduct was found to be as toxic as the parent compound for two of the organisms tested, *B. calyciflorus* and *T. platyurus*, whereas no effect was found for *D. magna*.

Chronic tests showed higher toxicity than acute tests (**Table 2**). From these data it was possible to note that carboxin was bioactive at low concentrations mainly for the primary consumer *C. dubia* (0.64 mg/L), whereas it was 1 order of magnitude less inhibitory toward algae (2.41 mg/L). No toxic potential for sulfoxide **2** was evidenced for algae at the maximum concentrations tested of 30 mg/L, whereas it showed an activity similar to that of carboxin toward the crustacean. No phototransformation of carboxin and sulfoxide was found at the end of the experiments with algae, after 3 days of test solution exposure at 10000 lx.

In conclusion, carboxin is photodegraded by exposure to sunlight in water and, as found in organic solvents, is particularly sensitive to photo-oxidation conditions. Eight photoproducts have been isolated and characterized, confirming some previously reported results (*8*), and their pathways have been rationalized. The main product, which is the more photostable and less hydrolyzable, is sulfoxide **2**. It is noteworthy that carboxin is eventually oxidized to the sulfoxide **2** in soil or in various plant species and animals, too (*21*, *22*). The metabolite has non-fungitoxic activity and, as shown by our investigation, exhibits similar or even lower acute toxicity toward aquatic organisms.

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