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Removal and Toxicological Response of Triazophos by *Chlamydomonas reinhardtii*

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Algae, because of their substantial biomass and extensive range of habitat and diversity, play an important role in the fate of organic compounds in the aquatic ecosystem (Pflufmacher et al., 1999). They may degrade or take up contaminants, thereby acting as a medium for bioconcentration and subsequent biomagnification in higher trophic levels (Sijm et al.,1998) . Biosorption of organic pollutants by algae has been frequently reported (Larsson et al., 2000) , and some macroalgae have been shown to have the same detoxification enzymes as those found in the mammalian liver, the so-called green liver (Mason et al., 1996) . It has been demonstrated that *Ochromonas dancia* can metabolize phenolic compounds to pyuvate and carbon dioxide (Semple et al., 1999).

Triazophos (*O,O-diethyl-O-1-phenyl-1H-1,2,4-triazol-3-yl-phosphorothioate*, CAS Registry No. 24017-47-8) is one of the midtoxic, broad spectrum, nonsystemic, and contact organophosphorus pesticides (OPs), and it has been put into agricultural use since the late 1970s. In recent years, most high-toxic and high-residual OPs were banned for use on crops by the agricultural department of China. As a good alternative, less toxic triazophos has been extensively applied in China, which raises a great concern about its environmental behavior. The hydrolytic kinetics of triazophos under different pH and temperature conditions have been investigated by Kunde et al. (2004) . Ma et al. (2004) studied the different responses of four cyanobacterial and green algal species exposed to triazophos. Additionally, Rani et al. (2001) reported the persistence and dissipation behavior of triazophos in canal water under Indian climatic conditions. The microbial degradation of organic pollutants in the aquatic environment has been studied for more than 30 years; these studies mainly focused on the role of bacteria and fungi (Semple et al., 1999). However, few data are available on the role of algae. The aim of this study is to investigate the removal of triazophos, including biodegradation and biosorption, by *Chlamydomonas reinhardtii* and to investigate the toxicological response of triazophos to the green algae that is widely present in the aquatic system.

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Materials and Methods

Triazophos, with 98.5% purity, was purchased from China Quality Testing Center of Pesticide (Shenyang, Northeast China). 1-aminoben-zotriazole (ABT) was purchased from Sigma Chemical Co., Ltd. (USA). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific Co., Ltd. (United Kingdom). The reagents used in this study (anhydrous sodium sulfate, sodium chloride, ethyl acetate, chloroform, and n-hexane) were of analytical grade and were purchased from Shanghai Chemical Reagents Co., Ltd. (Shanghai, China).

An axenic culture of *C. reinhardtii* was obtained from the Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). *C. reinhardtii* was cultured in CHU-11 medium (www.Index of Cyanobacteria Medium

Recipes for Modern Browsers) in 250 mL Erlenmeyer flask at 25° under illumination of 45 $\mu Em^{-2}s^{-1}$ with a 12:12 h light and dark shift cycle in an environmental chamber. The pH of the medium varied between 7 and 8, and was not adjusted over the whole experimental period (8 d).

C. reinhardtii cells at exponential growth phase were inoculated into CHU-11 medium (100 mL) and were diluted to a cell density at 1×10^5 cell/mL. An appropriate amount of triazophos (EC_{50}, 36 mg/L) stock solution was added to the preceding medium, and the final concentration of 10 μg/mL was obtained. In order to determine whether cytochrome P_{450} was involved in the biotransformation of triazophos, the effect of specific cytochrome P_{450} inhibitors (ABT) on triazophos removal by *C. reinhardtii* was also investigated. The EC_{50} of ABT on *C. reinhardtii* was 48 μM. The following treatments were set up: (a) medium amended with traizophos (10 μg/mL); (b) medium amended with traizophos (10 μg/mL) and algal cells (1×10^5 cell/mL), and (c) medium amended with traizophos (10 μg/mL) and fortified with the mixture of 30 μmol/L ABT and algal cells (1×10^5 cell/mL). The treatment for medium spiked with triazophos without algal cells served as a control. Each set was in triplicate and incubated for 8 d under the same conditions as described previously. At the fixed intervals of 0, 2, 4, 6, and 8 d after treatment (DAT), a 10-mL sample was collected and was processed for residue analysis of triazophos.

Aliquots (10 mL) of algal suspension at different intervals were first filtered with a 0.22-μm membrane, then were introduced into a separatory funnel (60 mL), and 2 g of sodium chloride was added to it (Jain et al., 1987). The triazophos residue was extracted by partitioning of the medium three times with ethyl acetate (5 mL each time). The organic layer was collected over anhydrous sodium sulfate, and its volume was reduced to 5 mL with a rotary vacuum evaporator for estimation by GC. The density of algal cells in the culture was determined using a hematocytometer, and time-trend of the growth rate was also monitored over the whole experimental period (8 d).

To estimate the recovery of triazophos residue in algal cells, a recovery study was performed by preparation of 40-mL algal suspension at an initial density of 20×10^5 cells/mL for each set. The former algal suspension was centrifuged at 3000 rpm for 30 min, then the supernatant removed and the remaining cell pellet was transferred into the beaker fortified with triazophos stock solution to obtain the final concentrations of 1, 3, and 5 μg/g, respectively. The samples were placed in the dark for 1 hr, and the mixing solvent of ethyl acetate and n-hexane (1/1 by volume) was used to extract triazophos residue. The algal cells were crashed under ultrasonic conditions at 50 rpm for 4 min with 60-s intervals for cooling the sample. The supernatant was used as a source of cell-free extract for further clean-up process.

The former supernatant was transferred into a glass column (1.5 cm i.d. × 15 cm length) packed with a mixture of active carbon (28 mg) and anhydrous sodium sulfate (5 g), and was followed by rinse with 4 mL mixing solvent of ethyl acetate and n-hexane (75/25 by volume) and 6 mL mixing solvent of ethyl acetate and n-hexane (50/50 by volume). The eluate was evaporated to dryness on a rotary vacuum evaporator, and the residue was redissolved in ethyl acetate (5 mL) for GC analysis.

An Agilent 6890N series GC (Agilent Technologies, USA), equipped with NPD detector, was performed to determine the residue of triazophos and a capillary column (30 m × 0.32 mm i.d. × 0.25 μm film thickness) used. The operating conditions were as follows: injection port temperature, 230°; splitless mode; injection volume, 1 μL; column temperature program, 160°—20°/min —240° (3 min); flow rate, N_2 5 mL/min; make up gas + column flow 10 mL/min; H_2 3 mL/min; air 60 mL/min; detector temperature 320°.

The preliminary experiment proved that the growth rate of *C. reinhardtii* in medium was not affected by the addition of 10-μg/mL traizophos. To further ascertain the effect of triazophos on *C. reinhardtii* antioxidant enzymatic activity, the activities of the following enzymes—superoxide dismutase (SOD), peroxidase (POD), and organophosphorus hydrolase (OPH)—were determined in the algal cells after 96-hr exposure to traizophos.

Cells from the control and treatment samples (96-hr old) were harvested by centrifugation at 10,000 rpm for 10 min at 4°. The cell pellet was resuspended with 10 mL 0.1 M sodium phosphate (pH 7.6) containing 0.1% Triton X-100 and was transferred onto the glass bead surface of a bead-beater. Additional buffer (0.8 mL) was added to top off the beads to ensure that the suspension was uniformly distributed. The bead-beater was placed in an ice bath and was allowed to cool for 5 min. The sample was homogenized for three 20-s bursts with 30-s intervals to allow cooling. The homogenate with beads was then transferred into the centrifugation tubes with 4 mL sodium phosphate buffer (pH 7.6). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°, and the resulting pellet was discarded. The supernatant was used as a source of cell-free extract for use in enzyme activity assays. The activities of SOD, POD, and OPH were determined according to the method reported by Beyer and Fridovich (1987), Putter (1975), and Mulchandani et al. (1999), respectively.

Results and Discussion

The recoveries of triazophos in medium and in algal cells are presented in Table 1 and Table 2, respectively. As can be seen in Table 1, the average recoveries were in the

Table 1 Recoveries of triazophos in CHU-11 medium and algal cells

Matrices	Spiking level (μg/mL)	Recovery (%)				
		R_1	R_2	R_3	Average±SD	RSD (%)
CHU-11 medium	0.5	79.3	81.7	83.2	81.4±2.0	2.4
	5	97.1	89.3	91.5	92.6±4.0	4.3
	10	90.0	84.9	86.4	87.1±2.6	3.0
Algal cells	1	78.3	77.5	80.2	78.7±1.4	1.8
	3	82.5	83.8	83.1	83.1±0.6	0.7
	5	93.3	99.0	92.4	94.9±3.6	3.8

range of 81.4%–92.6%, and the relative standard deviations (RSD) ranged from 2.4%–4.3% in CHU-11 medium. Similarly, the average recoveries in algal cells varied between 78.7% and 94.9%, and RSD varied from 0.7%–3.8%, respectively. Therefore, the method adopted for analyses of triazophos residues in medium and in algal cells was both satisfactory.

The effect of triazophos, at an initial spiking level (10 μg/mL), on the growth rate of *C. reinhardtii* is shown in Fig. 1. By comparison with the control, the addition of triazophos did not significantly affect the growth of algae, because the spiking level was much lower than the EC_{50} (36 μg/mL) of triazophos on *C. reinhardtii*. The concentrations of triazophos declined gradually with time in control medium, whereas it declined sharply from 2 DAT in *C. reinhardtii* culture (Fig. 2). An additional interesting finding was that the removal rate for *C. reinhardtii* treatment at 0 DAT (1 hr after spiking) was more than 40%, which proved that this algae had a high adsorbility to triazophos. The dissipation of triazophos over 8 d in the control medium was 33.4%, but it was below detection level (<0.005 μg/mL) in *C. reinhardtii* culture. For the control, the removal (33.4%) was mainly involved in the chemical mechanism including photolysis and hydrolysis, whereas the additional removal of triazophos in the *C. reinhardtii* culture (about 66.6%) may be caused by factors such as adsorption to the cell surface, biodegradation, biotransformation, or mineralization by *C. reinhardtii*. To differentiate the proportion of triazophos being (a) adsorbed or (b) biodegraded and biotransformed by *C. reinhardtii*, triazophos residues in *C. reinhardtii* cells were further determined. Results showed that the amount of triazophos adsorbed by *C. reinhardtii* cells was 22.7% of the initially spiking level, which accounted for 34.1% of the total loss (66.6%) by *C. reinhardtii* except for the degradation occurring in the chemical mechanism. Thus, it could be concluded that the remaining removal, 43.9% of the initial concentration, was caused by biotransformation or biodegradation by *C. reinhardtii*, which accounted for 65.9% of the total loss concerned in the biological mechanism. In toxicology, the bioconcentration factor (BCF) is defined as the ratio between the residual concentration in the organism and the concentration in the environment. As a result, BCF was estimated to be about 142, which further proved that *C. reinhardtii* has a high biosorption to triazophos. BCF can be determined experimentally or deduced from physicochemical properties of the chemical, such as the n-octanol/water partition coefficient (K_{ow}) or water

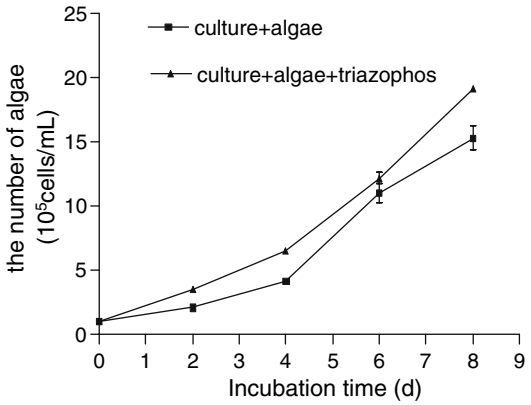

Fig. 1 The effect of triazophos (10 mg/L) on *C. reinhardtii* growth

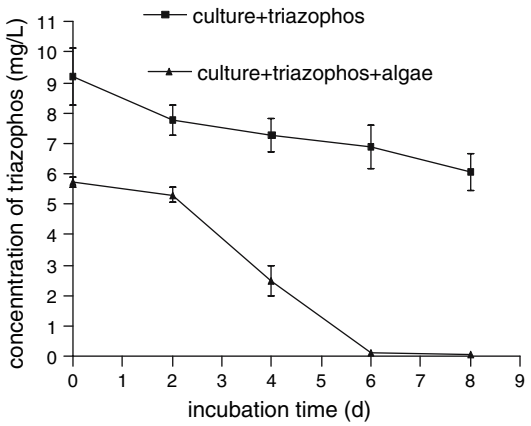

Fig. 2 Removal of triazophos with incubation time by *C. reinhardtii*

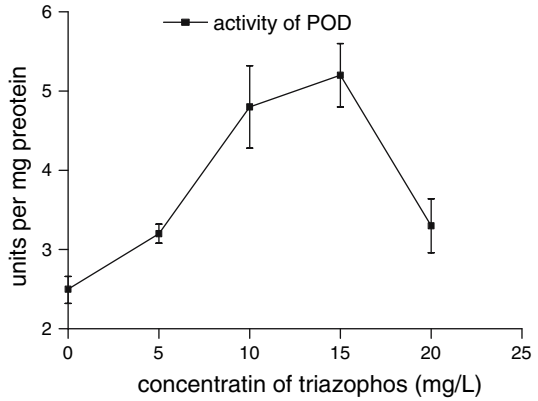

Fig. 3 Activity of POD when *C. reinhardtii* is exposed to triazophos

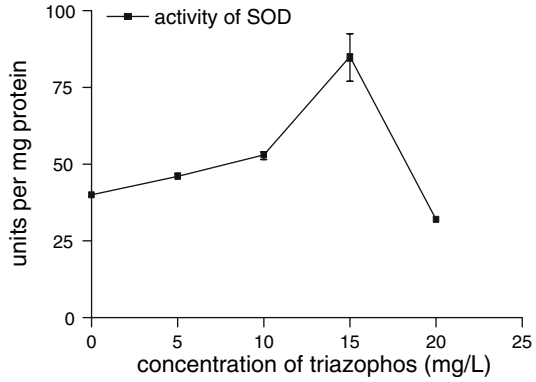

Fig. 4 Activity of SOD when *C. reinhardtii* exposed to triazophos

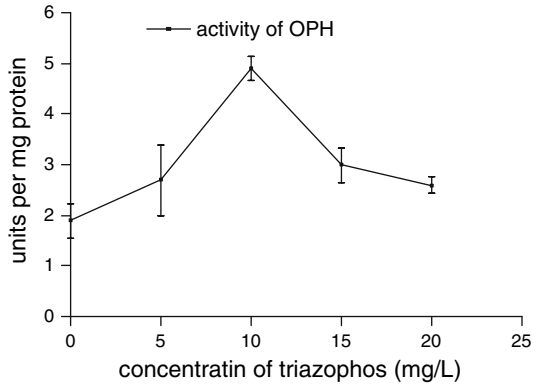

Fig. 5 Activity of OPH when *C. reinhardtii* exposed to triazophos

solubility, and BCF is often influenced by a variety of physiological and environmental conditions of the organisms (Kirk et al., 1999). In this study, the high BCF (142) in *C. reinhardtii* cells might be caused by the high log K_{ow} (log K_{ow} = 3.3) of triazophos in fresh water on the basis of Crosa's reports (1995) that high BCF values (log K_{ow}) were expected for pesticide showing 3 < logKow < 6.

Also, the biodegradation process of triazophos in the *C. reinhardtii* culture fit the first-order kinetics equation ($\ln(C_t/C_0)$ = 0.1497t–0.3751, R^2=0.99174) with a half-life of 4.6 d. In brief, not only had *C. reinhardtii* the ability to adsorb but also to degrade or transform triazophos. 34.1% of the total loss involved in the biological mechanism was a result of the adsorption by *C. reinhardtii* cells, whereas the remaining (65.9%) was attributed to the biodegradation mechanism.

The presence of ABT had a significant effect on triazophos removal, which showed that the addition of the specific cytochrome P450 inhibitors clearly decreased the removal rate (only 9%) by *C. reinhardtii* (data not shown in Fig. 2) at the conclusion of the experiment (8 d). However, the removal rate reached approximately 66.6% in the absence of ABT, which stongly suggested that cytochrome P450 was involved in the biodegradation of triazophos.

A short-term (96 hr) experiment was conducted to investigate the response of antioxidant enzymes when *C. reinhardtii* was exposed to triazophos. POD is an important component of the resisting oxygen system by means of clearing off $O_2^{\bullet}$and OH^{-1} in plants and organisms, which plays an important role in resisting the adverse circumstances. As shown in Fig. 3, POD activity increased gradually when the trizophos fortification level ranged from 5–15 mg/L. The highest activity of POD was observed at 15-mg/L triazophos, which increased about 2.5-fold over that for the control. However, the sharply declining trend was observed when *C. reinhardtii* was exposed from 15–20 mg/L, which indicated that POD activity was severely inhibited. The preceding results demonstrated that *C. reinhardtii* could clear the harmful matter by increasing POD activity for the lower triazophos level. However, the higher concentration would inhibit POD activity. In brief, the role of *C. reinhardtii* in clearing the pollutions by increasing POD activity was limited.

SOD, along with POD, compose the protection enzymatic system, which are the important enzymes for an organism to accommodate many kinds of adverse circumstances. The similarly increasing and declining trends for SOD activity as POD are observed (Fig. 4), and the critical concentration was also found to be about 15 μg/mL, at which concentration the SOD activity reached its peak (85 units/mg protein). The former results showed that the harm to *C. reinhardtii* caused by triazophos could be eliminated by increasing SOD activity. However, the continuous fortification of triazophos led to the sharp decline of SOD activity, which was only 26 units/mg protein and was lower than that for control when exposed to 20-μg/mL triazophos. By comparison with SOD and POD, OPH activity reached its peak (4.9 unit/mg protein) when *C. reinhardtii* was exposed to triazophos at 10 μg/mL, and then declined gradually (Fig. 5). It was apparent that OPH was more sensitive to triazophos than SOD and POD.

In conclusion, triazophos could be, to some extent, removed through biodegradation and biosorption in *C. reinhardtii* culture. *C. reinhardtii* could remove triazophos by 66.6% at 8 DAT, which showed an important application value in bioremediation of triazophos-contaminated waters. *C. reinhardtii* could decrease the toxicology of triazophos by increasing POD and SOD activities at the lower fortification level. However, the higher concentration would severely inhibit two enzymatic activities. As compared with SOD and POD, OPH was found to be more sensitive to triazophos.

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