SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 27, No. 1, pp. 168–173, 2008
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Printed in the USA
0730-7268/08 $12.00 + .00

Environmental Research in China

RESPONSE OF THE FRESHWATER ALGA *CHLORELLA VULGARIS* TO TRICHLOROISOCYANURIC ACID AND CIPROFLOXACIN

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(*Received* 11 *January* 2007; *Accepted* 21 *June* 2007)

Abstract—The effects of trichloroisocyanuric acid (TCCA) and ciprofloxacin (CPFX) on the freshwater alga *Chlorella vulgaris* were assessed by toxicity bioassays and by the values of biomarkers in phase I and phase II. The biomarkers included growth rate, concentration of chlorophyll *a*, activities of 7-ethoxyresorufin-*O*-dealkylases (EROD), glutathione *S*-transferase (GST), catalase (CAT), and total glutathione (GSH). Ciprofloxacin was a weaker growth inhibitor than TCCA but, at a concentration of greater than 12.5 mg/L, decreased the growth of *C. vulgaris*. Concentration of chlorophyll *a* showed a similar trend. The 96-h median effective concentration (EC50; i.e., 50% reduction in growth relative to the control) of CPFX was 20.6 mg/L. Trichloroisocyanuric acid was a strong growth inhibitor and, at concentrations of greater than 0.80 mg/L, caused 100% inhibition on 24-h exposure. The 96-h EC50 of TCCA was 0.313 mg/L. Ciprofloxacin and TCCA affected the phase I and phase II enzyme activities differently. On exposure to CPFX, both EROD and GSH decreased at low CPFX concentrations (<5.0 mg/L) and increased at high CPFX concentrations (>12.5 mg/L), and CAT and GST exhibited induction at low concentrations and inhibition at high concentrations. In TCCA exposure, GST activity was significantly stimulated, and GSH concentration was increased. Catalase activity increased only at TCCA concentrations of greater than 0.12 mg/L, and no change in EROD activity was observed.

Keywords—Trichloroisocyanuric acid Ciprofloxacin *Chlorella vulgaris* 7-Ethoxyresorufin-*O*-dealkylases Antioxidant enzyme

INTRODUCTION

The environmental effects of pharmaceuticals, antibiotics, and disinfectants are of increasing concern [1–7]. Once released into the environment, these xenobiotics may bring about deleterious effects on sensitive microorganisms. As a consequence, the effect on microorganisms such as algae will extend to the whole ecosystem. Therefore, the potential impact of these xenobiotics on the ecosystem must be evaluated as closely as that of other hazardous chemicals.

Photoautotrophic microalgae are the primary producers in the ecosystem. They are on the lowest level of the nutrient pyramid and constitute the foundation of the entire food web in an aquatic ecosystem. Consequently, toxicity to these organisms is of particular importance. Potential toxic effects of pharmaceuticals, antibiotics, and disinfectants, however, have not been properly investigated and evaluated, even though these compounds are widely used in aquatic ecosystems. Research has focused mostly on the effects of herbicides on algae [8–10]. Less than 1% of the ecotoxicological data concerns pharmaceuticals [2].

Trichloroisocyanuric acid (TCCA) is a disinfectant used widely in hospitals, hotels, restaurants, swimming pools, industrial cooling water, and aquaculture because of its effective biocidal activity against bacteria, viruses, and fungi. Trichloroisocyanuric acid is an unstable chemical and forms hypochlorous acid (HClO) and hypochlorite ions (ClO^-), which are effective biocides in contact with water. In addition, hypochlorous acid and hypochlorite ions can react with organic compounds by addition, substitution, and oxidation and can produce toxic chlorinated by-products that are very stable and toxic to aquatic organisms [11]. Van Wijk et al. [12] reported that chlorite, a potential toxic metabolite of chlorate, showed high toxicity to different taxa, including plants, bacteria, and fungi. Data regarding the toxicity of chlorinated disinfectants to algae, however, are limited [12].

Ciprofloxacin (CPFX), a third-generation quinolone antibiotic, is effective against aerobic Gram-negative and Gram-positive bacteria. At present, it is universally used in medical treatments, farming, and aquaculture because of its very broad spectrum of antibacterial effects and low price. It kills bacteria by inhibiting the activity of DNA gyrase and by interfering with the copying of DNA. In plants, the target of CPFX may be DNA–topoisomerase II, analogous to the bacterial gyrase [13]. It is reported that the median effective concentration (EC50) and no-observable-effect concentration of another quinolone antibiotic, norfloxacin, for *Chlorella vulgaris* are 10.4 and 4.1 mg/L, respectively [14]. Enrofloxacin, a quinolone used widely in farming, was stored in plants and induced some adverse effects on their growth [13]. Flumequine (another quinolone) also was taken up by the aquatic weed *Lythrum salicaria* L., was bioaccumulated under laboratory conditions, and was phytotoxic [15,16]. Very little is known about toxic effects of CPFX on the aquatic alga *C. vulgaris*.

The metabolism of pharmaceuticals and related xenobiotics in plants is similar to that in animals, including phase I transformation, phase II conjugation, and phase III compartmentalization. In both, the cytochrome P450 (CYP450) enzyme system and the antioxidant defenses system play a very im-

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Published on the Web 9/14/2007.

portant role [17]. A large body of literature is available concerning the response of CYP450 mono-oxygenase enzymes and antioxidative enzymes in fish and mammals to pollutants [18,19]. 7-Ethoxyresorufin-*O*-deethylase (EROD), a marker of CYP450 enzyme system activity, is a well-documented biomarker of exposure to many xenobiotics in animals [20,21]. Glutathione *S*-transferase (GST) and catalase (CAT) are two enzymes in the phase II pathway that are useful biomarkers for the oxidative stress frequently induced by xenobiotics [4].

Little information is available regarding the activities of the phase I transformation enzymes and phase II conjugation enzymes in lower plants, such as algae, exposed to pharmaceuticals, particularly chlorinated disinfectants and quinolone antibiotics. The objective of the present work was to study the ecotoxicity of a typical pharmaceutical (CPFX) and a common disinfectant (TCCA) on the green alga *C. vulgaris* as judged by growth inhibition and the response of phase I and phase II enzymes.

MATERIALS AND METHODS

Plant materials

The unicellular alga *C. vulgaris* was provided by the Algal Species Conservation Center of the Hydrobiology Institute of Jinan University in China. *Chlorella vulgaris* was chosen for the present study because of its widespread distribution in freshwater ecosystems and its frequent use for growth-inhibition toxicity tests. The alga was routinely cultivated in our laboratory under standardized abiotic conditions, continuous illumination provided by cool-white fluorescent light, temperature of 22 ± 1°C, and permanent aeration in orbital shaker (130 rpm). Algal cultures used for the experiments were maintained in the exponential growth phase, and stock sample had a cell density of 1.2×10^7 cell/ml.

Experimental treatments

Trichloroisocyanuric acid was purchased from Fluka (Steinheim, Germany) and CPFX from Sigma (St. Louis, MO, USA). Stock solutions were prepared by dissolution in ultrapure water (Millipore, Shanghai, China). The algal cultures were exposed to a series of concentrations (0.00, 0.05, 0.12, 0.32, and 0.80 mg/L of TCCA and 0.00, 2.00, 5.00, 12.25, and 31.25 mg/L of CPFX) for 24, 48, 72, and 96 h. Concentrations of CPFX and TCCA were measured by high-performance liquid chromatography, and active chlorine in the TCCA samples was performed using the iodometric method at 0, 24, 48, and 96 h in solutions without algal cells [22]. In the toxicity experiments, three replicates per concentration were performed.

Growth rate determination

The growth rate was measured by directly counting the number of cells in the hemocytometer at 24, 48, 72, and 96 h after inoculation. The growth inhibition rate was calculated according to the Organization for Economic Cooperation and Development guideline [23]:

$$\mu_{t_i} = \ln(N_{t_i}/N_0)/(t_i - t_0) \qquad \rho_{t_i} = 100(\mu_0 - \mu_{t_i})/\mu_0$$

where N_{t_i} is the cell count at time t_i, N_0 is the initial cell count, μ_{t_i} is the relative growth rate at time t_i, μ_0 is the growth rate of control treatment, and ρ_{t_i} is the inhibition ratio.

Chlorophyll determination

A sample of the algal culture (50 ml) was collected at 24, 48, 72, and 96 h after inoculation and centrifuged at 10,000 *g* for 10 min. Collected algae were weighed and ground with quartz powder and 1 ml of acetone. Chlorophyll was extracted by acetone, and its concentration was determined spectrophotometrically [24].

Enzyme extraction

A sample of the algal culture (50 ml) was collected at 96 h and centrifuged at 5,000 *g* for 10 min, after which the supernatant was discarded. Collected algae were washed twice with 5 ml of buffer (50 mM potassium phosphate and 150 mM potassium chloride, pH 7.5). The algae were then transferred into 5 ml of the buffer solution, ground at 4°C with quartz powder, and then centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant containing the enzymes was stored at −80°C for assays [4].

Enzyme measurements

The EROD level was determined at the excitation wavelength of 550 nm and the emission wavelength of 580 nm with spectrofluorometry as described by Pohl and Fouts [25]. The GST was evaluated by the change of absorbance at 340 nm because of the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene, as described by Habig et al. [26]. Catalase activity was detected by measuring the decrease in absorbance at 240 nm at 25°C for 1 to 6 min [27]. Total GSH concentration was determined by 2,5-dithiobis-tetranitrobenzoic acid and oxidized glutathione reductase recycling assay [28]. Protein content was measured following an adaptation of the procedure described by Bradford [29].

Statistical analysis

All exposure experiments were repeated three times, and each sample was analyzed in triplicate. Differences between controls and treatments were determined using a one-way analysis of variance (ANOVA), with $p < 0.05$ considered to be significant.

RESULTS

Toxicant concentrations

The concentration of CPFX remained practically constant during the experiment at 83 to 91% of the nominal concentration. The concentrations of TCCA decreased according to pseudo–first order kinetics, with rate constants of 136, 58, 49, and 47/h at the nominal concentrations of 0.05, 0.12, 0.32, and 0.80 mg/L, respectively (Fig. 1).

Growth inhibition and chlorophyll a *content*

Ciprofloxacin is a weak inhibitor of *C. vulgaris*. The cell growth was not reduced significantly with the increase of CPFX concentration at an exposure time of less than 48 h. At longer exposure times, however, the growth of *C. vulgaris* was inhibited by CPFX at all concentrations, with a typical concentration–response curve. Compared to the control treatment, concentrations of 2.0 and 31.25 mg/L resulted in a growth inhibition rate of 9.2 and 72.4% of control after 96-h exposure of CPFX, respectively (Table 1). Chlorophyll *a* content showed a similar trend. Exposure of *C. lorella vulgaris* for 48 h showed no difference from control in chlorophyll *a* content at all exposure concentrations; however, the highest concentration (31.25 mg/L) resulted in a 51.5% decrease after 96 h of exposure (Fig. 2).

A TCCA concentration of 0.80 mg/L inhibited the growth

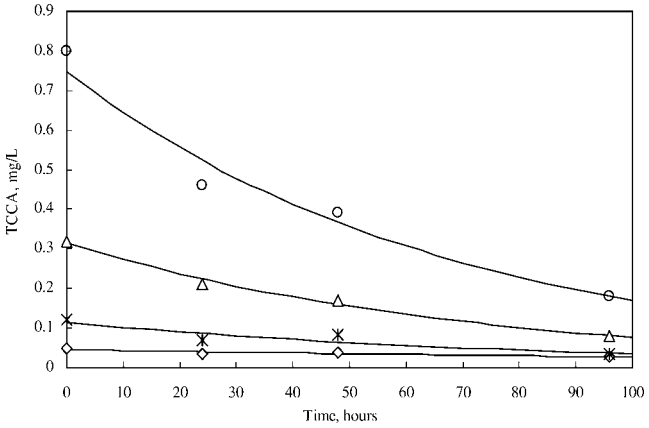

Fig. 1. The kinetics change of the concentration of trichloroisocyanuric acid (TCCA) with time in solutions without *Chlorella vulgaris*. Concentration of TCCA were 0.05 (◇), 0.12 (✳), 0.32 (△), and 0.80 mg/L (○).

of *C. vulgaris* by 79.2% after 24 h of exposure (Table 2). *Chlorella vulgaris* cells started dying after 48 h. Many broken cells could be observed under the microscope. Low concentrations (0.05–0.12 mg/L), however, had no effect on the growth of *C. vulgaris* during the entire exposure period. On the contrary, exposure of *C. vulgaris* to low concentrations of TCCA stimulated growth to some extent, resulting in a negative growth inhibition rate (Table 2). Similar response was observed in chlorophyll *a* content. A concentration of 0.32 mg/L caused a 37.3% decrease in chlorophyll *a* content after 96 h of exposure (Fig. 2). The 96-h EC50 of CPFX and TCCA for *C. vulgaris* were 20.6 and 0.313 mg/L, respectively.

Enzyme activities

Phase I and phase II enzyme activities were affected differently in *C. vulgaris* by CPFX and TCCA. After exposure to CPFX, EROD activity and GSH concentration decreased with the increase of CPFX during the lower range of concentrations, with a minimum value of 4.24 pmol/min/mg protein and 103.6 mg/g protein, respectively, at the concentration of 12.5 mg/L of CPFX, and then increased at 32.5 mg/L of CPFX (Fig. 3). On the contrary, CAT and GST activities increased with increasing CPFX concentration and declined at 32.5 mg/L of CPFX.

During the exposure to TCCA, the activity of GST and the concentration of GSH were significantly increased at a TCCA concentration of 0.32 mg/L after 96 h of exposure. No significant changes in the activity of EROD were observed during the entire exposure period. At low concentrations of TCCA (0.05 and 0.12 mg/L), no dose-dependent relationship was observed for CAT, but at a TCCA concentration of 0.32 mg/L, CAT activity increased to 38.7-fold that of the control. Algal growth was inhibited at a TCCA concentration of 0.8 mg/L, and algal cell amounts were too low to be collected. 7-Ethoxyresorufin-*O*-deethylase, GST, CAT, and GSH could not be measured.

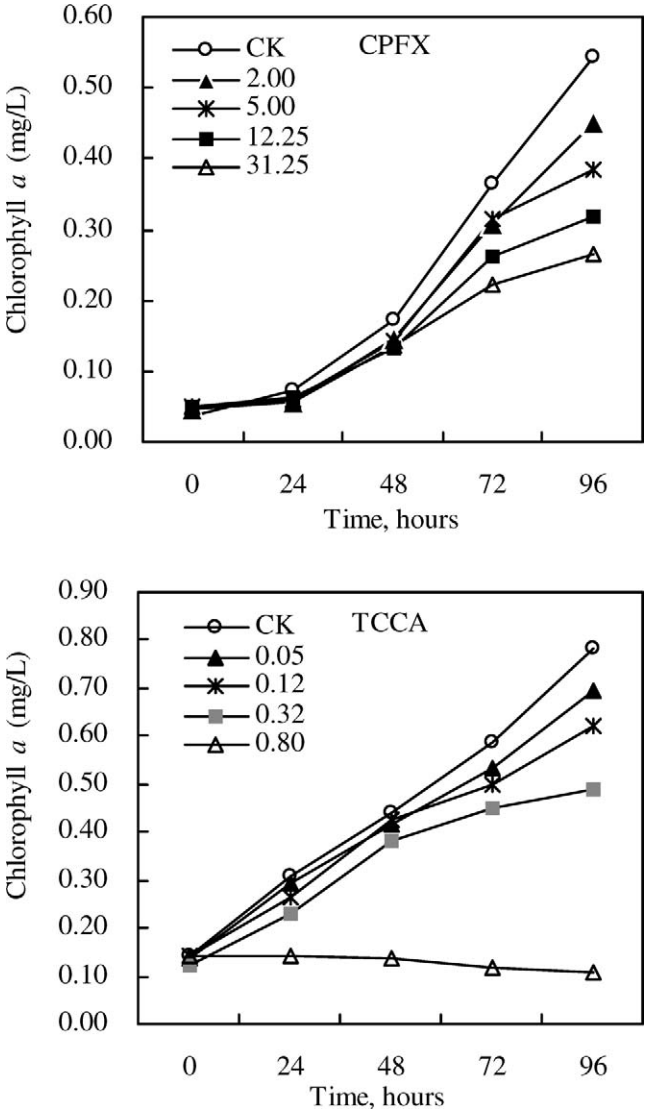

Fig. 2. The effects of trichloroisocyanuric acid (TCCA) and ciprofloxacin (CPFX) on the content of chlorophyll *a* in *Chlorella vulgaris*. CK = control treatment group.

DISCUSSION

The EC50 of CPFX for *C. vulgaris* as determined in the present study is higher than the EC50s reported in the literature

Table 1. Growth inhibition ratio of *Chlorella vulgaris* as a function of ciprofloxacin concentration and length of exposure[a]

Concentration (mg/L)	ρ_{t_i} (%)[b]			
	24 h	48 h	72 h	96h
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.00	4.26 ± 0.12*	6.36 ± 0.21*	8.13 ± 0.15*	9.24 ± 0.31*
5.00	8.76 ± 0.19*	12.70 ± 0.23*	17.16 ± 0.26*	20.55 ± 0.18**
12.50	12.21 ± 0.09*	18.16 ± 0.13*	32.07 ± 0.32**	38.60 ± 0.24**
31.25	16.18 ± 0.22*	26.42 ± 0.41**	54.89 ± 0.27**	72.40 ± 0.33**

[a] Values are presented as the mean ± standard deviation. *$p < 0.05$, **$p < 0.01$.
[b] ρ_{t_i} = inhibition ratio.

Table 2. Growth inhibition ratio of *Chlorella vulgaris* as a function of trichloroisocyanuric acid concentration and length of exposure[a]

Concentration (mg/L)	ρ_{I_i} (%)[b]			
	24 h	48 h	72 h	96 h
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.05	−9.59 ± 0.07*	−0.15 ± 0.09	−2.89 ± 0.12	0.03 ± 0.02
0.12	1.14 ± 0.08	4.40 ± 0.15	1.87 ± 0.16	6.38 ± 0.19*
0.32	1.30 ± 0.11	6.67 ± 0.13*	7.30 ± 0.21*	9.13 ± 0.23*
0.80	79.18 ± 0.27**	108.32 ± 0.24**	137.55 ± 0.29**	113.63 ± 0.31**

[a] Values are presented as the mean ± standard deviation. *$p < 0.05$, **$p < 0.01$.
[b] ρ_{I_i} = inhibition ratio.

for CPFX and other quinolones. Ciprofloxacin used in the present experiments is the hydrochloride, the solubility of which in water is affected by the change of pH [30]. With algal cell growth, the pH in medium increases appreciably, and this may decrease the solubility of CPFX and, thus, its effective concentration in the medium. The lower toxicity of CPFX to *C. vulgaris* also may relate to some ingredients in algal culture medium (e.g., magnesium ions). Divalent cations such as magnesium may lead to an antagonistic effect and depress the bioavailability of CPFX for aquatic organisms [31]. It is possible that Mg^{2+} mediates drug–DNA interaction. The amount of quinolone bound to DNA is modulated by the concentration of Mg^{2+} [30]. Also, quinolones interfere with the catalytic cycle of bacterial gyrase by stabilizing the gyrase–DNA cleavable intermediate, and gyrase needs the magnesium ion as a cofactor to perform its catalytic activity [32].

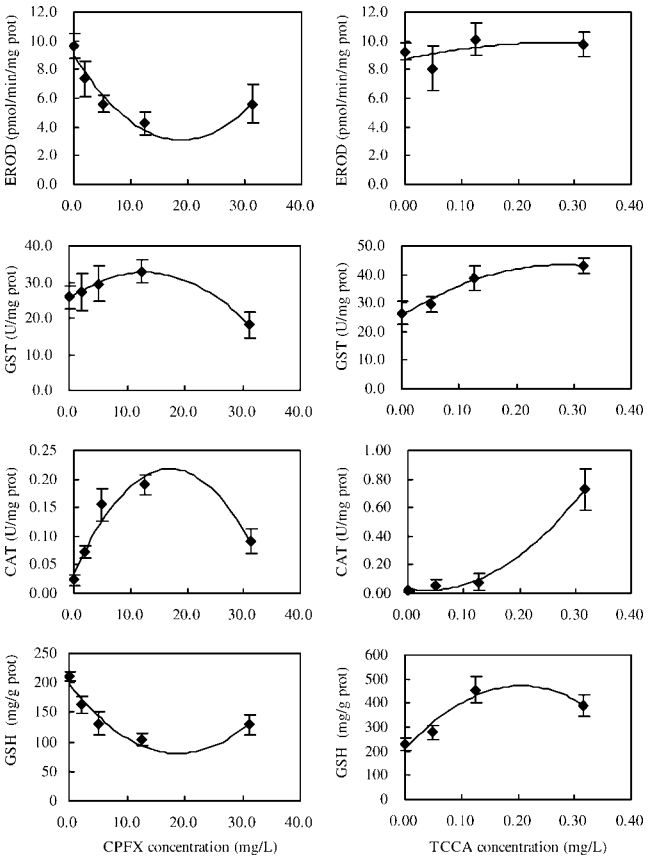

Fig. 3. The effects of trichloroisocyanuric acid (TCCA) and ciprofloxacin (CPFX) on the enzymes activity of *Chlorella vulgaris*. CAT = catalase; EROD = 7-ethoxyresorufin-*O*-dealkylases; GSH = total glutathione; GST = glutathione *S*-transferase.

The toxicity of TCCA may be attributed to hypochlorite from TCCA. Hypochlorite is a strong oxidant that reacts rapidly with a wide range of biochemically important compounds, such as thiols and amino acids. Sulfhydryl and amino groups in proteins are the primary target of hypochlorite. Hypochlorite could modify proteins and enzymes, cause the inactivation of cytochromes in electron-transport systems, and induce oxidation of lipids [33]. The oxidative damage to intracellular sulfhydryl groups is a more likely mechanism. The depletion of glutathione may render the cell more susceptible to subsequent membrane damage, such as by iron released from the cell [34]. Stauber [33] reported that chlorate was toxic to some marine brown macroalgae, particularly in nitrate-limited waters. This may relate to chlorate being reduced to chlorite or hypochlorite by nitrate reductase. In the present study, TCCA showed strong inhibition for algal growth. The increased GST and CAT activity accompanied by the decrease of total GSH content in algae at high TCCA concentration indicated that TCCA, indeed, induces the production of reactive oxygen species (ROS).

The growth inhibition of algae is always related to the inhibition of chlorophyll biosynthesis and to the peroxidative destruction of thylakoid membranes under the exposure of toxic compounds. This also was confirmed in the present experiments, in which the change of algal growth exhibited very good comparability and synchronization with chlorophyll *a* content in exposures to CPFX and TCCA.

The CYP450 enzyme family comprises multiple isoforms with different substrate specificities and often is responsible for the metabolism of a large number of pharmaceuticals. In the present study, EROD activity decreased at low concentration and increased at high concentration of CPFX. Vaccaro et al. [20] reported that CPFX induced the inhibition of CYP450 mono-oxygenase activities in fish. This may be attributed to CPFX providing the oxidation site for CYP450 catalysis. The strained cyclopropane ring of CPFX may be the site attacked by the CYP450 system. This may lead to reactive intermediates responsible for suicide enzymatic reaction. As a consequence, some intermediates with electrophilic properties or free radicals may be formed and cause a series of subsequent deleterious reactions. The reason for the increase of EROD activity at high concentration of CPFX is not clear, however, and needs further research.

Reactive oxygen species can be generated by the CYP450 system in the biotransformation process of xenobiotics such as CPFX and similar pharmaceuticals. Therefore, the induction of CYP450 also often is associated with the change in cellular antioxidant defenses [22]. Glutathione *S*-transferase catalyzes the conjugation of a variety of electrophilic compounds to GSH. Catalase is one of the main antioxidant enzymes in high-

er plants. Simultaneous induction usually is observed after exposure to pollutants; however, no such relationship was observed in the present study. The activities of CAT and GST increased when *C. vulgaris* was exposed to CPFX and TCCA (Fig. 3). Catalase activity increased by 8.2-fold at 12.5 mg/L in the CPFX exposure, whereas the GST activity was induced slightly. A low concentration of TCCA did not induce CAT, although GST activity was significantly increased. These findings are in agreement with previously reported results on antioxidant enzyme activities of algae exposed to the herbicide flumioxazin [4].

The stimulation of CAT and GST is a response to enhanced cellular ROS concentration to cope with the rises in ROS and peroxides. Quinolones contribute to ROS formation by a CYP450-dependent reaction [35], and TCCA itself, as a strong oxidative agent, can lead to the formation of ROS. Sodium chlorite derived from TCCA results in a substantial depletion of intracellular glutathione and can cause oxidative stress in cells at high concentration [36]. Consequently, the exposure of algal cells to these pharmaceuticals is expected to activate the antioxidant enzymes.

Glutathione was affected differently by CPFX and TCCA. Its concentration decreased with increasing concentration of CPFX but increased with increasing concentration of TCCA. Glutathione is a nonenzymatic antioxidant in algae that could bind some compounds with electrophilic properties and avoid further toxicity of xenobiotics to the cell. The ROS derived from the metabolism of CPFX by the CYP450 enzyme system may lead to the activation of the enzymes γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) to increase, eventually, the synthesis of GSH [37]. Trichloroisocyanuric acid itself is an oxidant and may directly induce the synthesis of GSH.

In an aquatic ecosystem, CPFX was eliminated by adsorption on the sediment, but the biodegradation was slow [38]. It was reported that CPFX was not biodegradable in the closed-bottle test [39]. Although the half-life of these pharmaceuticals is short compared to other persistent organic pollutants and the EC50s of CPFX and TCCA for *C. vulgaris* are an order of magnitude higher than the environmental levels of these compounds, their potential adverse effects at lower concentrations cannot be completely neglected. These pharmaceuticals are continuously introduced into the environment, resulting in chronic exposure of aquatic organisms.

Acknowledgement—Financial support from the National Natural Science Foundation of China (40471118; U0633006) is gratefully acknowledged.

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