Toxicological Responses of *Chlorella vulgaris* to Dichloromethane and Dichloroethane

Shijin Wu,¹ Huaxing Zhang,² Xiang Yu,¹ and Lequan Qiu^{1,*}

¹College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, People's Republic of China.

²Ningbo Institute of Technology, Zhejiang University, Ningbo, People's Republic of China.

Received: January 30, 2013

Accepted in revised form: November 12, 2013

Abstract

The aim of this study was to evaluate the acute toxicity effects of dichloromethane and dichloroethane on *Chlorella vulgaris* at the physiological and molecular level. Data showed that the cell number, chlorophyll a, and total protein content gradually decreased with increasing dichloromethane and dichloroethane concentrations over a 96-h exposure. Lower doses of two organic solvents had stimulatory effects on catalase and superoxide dismutase activity. Malondialdehyde showed a concentration-dependent increase in response to dichloromethane and dichloroethane exposure. Electron microscopy also showed that there were some chloroplast abnormalities in response to different concentrations of dichloromethane and dichloroethane exposure. Real-time polymerase chain reaction assay demonstrated that dichloromethane and dichloroethane reduced the transcript abundance of *psaB*, whereas that of *psbC* changed depending on the toxicant after 24 h of exposure. Dichloromethane and dichloroethane affected the activity of antioxidant enzymes, disrupted the chloroplast ultrastructure, and reduced transcription of photosynthesis-related genes in *C. vulgaris*, leading to metabolic disruption and cell death.

Key words: *Chlorella vulgaris*; dichloroethane; dichloromethane; real-time PCR; toxicological response

Introduction

POLLUTANTS LIKE PESTICIDES, hydrocarbons, and heavy metals, as well as thermal and radioactive agents, can enter aquatic environments after direct or indirect release from industries, agriculture, and households (Fathi *et al.*, 2008). Due to their widespread industrial use, large quantities of organic solvents are discharged into freshwater ecosystems, and their levels have increased substantially over the last century (Nriagu and Pacyna, 1988; Penuelas and Filella, 2002). Dichloromethane and dichloroethane are either confirmed or suspected carcinogens or mutagens in humans. Both may have endocrine disrupting effects on aquatic species, and are listed as 2 of the 14 volatile organic compounds that are regulated by the Safe Drinking Water Act Amendments of 1986 (Trotsenko *et al.*, 2000). In Japan, national effluent standards have been established to regulate these chemical substances. However, their influence on aquatic organisms, as opposed to human health, has not been studied in detail. Indeed, while the concentrations of such substances may meet the current effluent standards for human safety, they may still exert deleterious effects on the natural aquatic ecosystem.

Algae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, have been widely used in ecological risk assessments to evaluate the effects of contaminants on biodiversity because they are sensitive to contaminants at environmentally significant concentrations (Stauber and Davies, 2000; Levy *et al.*, 2007; Qian *et al.*, 2008a, 2008b). Traditionally, most of our knowledge regarding the toxicity of organic solvent pollutants to freshwater algae has been based on the effects of a single tested compound on such biochemical parameters as median effective concentration (EC₅₀), chlorophyll levels, biovolume, cell count, and average cell size (Wilde *et al.*, 2006). However, in sites polluted with solvents, two or more organic solvents are often found together in the environment. Given that natural pools of water are normally polluted by a mixture of substances, these solvents may exert synergistic toxic effects (Shuhaimi-Othman and Pascoe, 2007). A few studies have been conducted to investigate the combined effects of organic solvents on plant species. The combined toxic effects of dichloromethane and trichloroethylene on *Chlorella vulgaris* NIES227, *Selenastrum capricornutum* NIES35, and *Volvoxella steinii* NIES545 have been reported (Glenn and Tara, 1988). For example, results have shown that the combination of dichloromethane and trichloroethylene synergistically cause cell lysis. Although the literature regarding the biochemical and physiological influence of dichloromethane and trichloroethylene on algae is extensive, to our knowledge, the effects of their interaction on plankton have not been assessed or

*Corresponding author: College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, People's Republic of China. Phone: +86-571-88320658; Fax: +86-571-88320884; E-mail: lqqiu@sohu.com

reported at the enzymatic, ultrastructural, and the transcriptional level.

psaB and *psbC* are target photosynthesis-related genes. *psaB* is part of the *psaA/B* operon of the chloroplast genome and encodes the photosystem I (PSI) reaction center protein. *psbC* encodes the chlorophyll-protein complex CP43, an integral membrane protein component of photosystem II (PSII), which is one of the interior transducers of excitation energy from the light harvesting pigment proteins to the photochemical reaction center. In this study, *C. vulgaris* was chosen as the representative green microalga to evaluate the toxic effects of dichloromethane and dichloroethane individually and in combination at the physiological (growth, antioxidant enzymes, subcellular structure) and gene transcription (photosynthesis-related genes: *psbA* and *psaB*) levels.

Materials and Methods

Algal strains and culture conditions

C. vulgaris was obtained from the Institute of Wuhan Hydrobiology, Chinese Academy of Science. Cells of *C. vulgaris* were propagated photoautotrophically in a 250-mL Erlenmeyer flask containing 100 mL of liquid Shuisheng-4 medium (Zhou and Zhang, 1989), kept on a rotator shaker (100 rpm) at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, and illuminated with cool-white fluorescent lights at a continuous light intensity of 2500 lx/cm^2 for 14-h light and 10-h dark daily cycles. The culture medium was sterilized at 121°C , 1.05 kg/cm^2 for 30 min (Ma and Liang, 2001). For cell experiments, 15-mL aliquots of the HB-4 medium containing green algal cells (initial cell concentration $8 \times 10^5/\text{mL}$) were distributed into sterile 50-mL Erlenmeyer flasks. The media of *C. vulgaris* were then treated with various pollutant concentrations (dichloromethane: 0, 221, 299, 403, 550, 735, 992 mg/L; dichloroethane: 0, 192, 260, 351, 472, 639, 863 mg/L), and incubated for 96 h on a rotary shaker (100 rpm) at a temperature of $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ under a continuous light intensity of 2500 lx/cm^2 . Cell counts were correlated with absorbance over time for 96 h using a Shimadzu UV-2401PC spectrophotometer. The cell densities of the cultures were monitored spectrophotometrically at 685 nm (OD_{685} , optical density at 685 nm). The regression equation between cell density ($y \times 10^5/\text{mL}$) and OD_{685} (x) was calculated as $y = 162.1x + 1.3463$ ($r^2 = 99.34\%$). Each treatment was replicated three times. Appropriate controls systems containing no pollutant were included in each experiment. Control and treated cultures were grown under the same temperature, photoperiod, and rate of agitation as the stock cultures. In each experiment, percentage inhibition values, relative to growth in the controls were calculated using spectrophotometric data (Glenn and Tara, 1988; Golden, 1995). The EC_{50} values or pollutant concentrations required to cause 50% reduction in growth were calculated using linear regression analysis of in (transformed pollutant concentration) versus percentage inhibition. All correlation coefficients were >0.90 and most were >0.95 .

Dichloromethane and dichloroethane solutions in the culture medium were prepared when cultures reached the mid-log phase. Treatment conditions were selected based on the effects of these compounds in combination on algal cell numbers in preliminary experiments assessing acute toxicity from 12 to 96 h. Triplicate cultures were prepared for each dichloromethane and dichloroethane concentration and

combination. Samples were taken after 6, 12, 24, 48, and 96 h for RNA extraction. Enzymes were extracted at 12, 24, 48, and 96 h.

Enzyme extraction and assays

To extract antioxidant enzymes, 50 mL of each culture was centrifuged at $12,000 \text{ g}$ for 10 min. Separated algal cells were ground in 1 mL of 20 mM phosphate buffer (pH 7.4) plus 0.1 g of white quartz sand in a chilled tissue grinder. The homogenate was centrifuged at $12,000 \text{ g}$ for 10 min at 4°C to obtain the supernate for the enzyme activity and lipid peroxidation assays. The superoxide dismutase (SOD) activity in the supernate was determined using WST-1, a water-soluble tetrazolium as the sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Dojindo Laboratories), as the indicator of superoxide radicals generated by xanthine oxidase and hypoxanthine in the presence of a range of concentrations of SOD (Alexander and Christine, 2000). One unit of SOD activity was defined as the amount of enzyme inhibiting 50% of WST-1 photoreduction. The activity of catalase (CAT) was measured using the method by Piero *et al.* (1980). One unit of CAT activity was defined as the amount of hydrogen peroxide (H_2O_2) degraded per minute at 25°C .

Assays of chlorophyll, malondialdehyde, and protein concentration

Chlorophyll a (chl a), chlorophyll b (chl b), and total chlorophyll (total-chl) contents were analyzed according to the method by Inskeep and Bloom (1985). Lipid peroxidation levels were determined by the malondialdehyde (MDA) content using the method by Zhang and Kirkham (1994). Activity of each enzyme was expressed relative to protein content. Protein concentration was determined by BCA assay (BCA protein kit; Sangon Company). Measurements were made on a microplate reader using a microwell plate protocol A_{590} and the manufacturer's instructions.

Electron microscopy analysis

Samples of the control and pollutant-treated algae were fixed for 2 h in a 0.05 M cacodylate buffer (pH 7.2) solution containing 2.5% glutaraldehyde and 4% paraformaldehyde. The samples were then fixed with 1.0% OsO_4 in the same buffer for 1 h and subsequently dehydrated in acetone and embedded in epoxy resin. For transmission electron microscopy (TEM), ultrathin sections (70–90 nm) were obtained using a Reichert Ultracuts ultramicrotome, stained with uranyl acetate and then by lead citrate, and observed under a JEM-1230 microscope (JEOL Ltd.). The percentage of cells that lost lamella or the number of starch granules was determined by analyzing all cells within 10 microscopic fields.

RNA extraction

To extract RNA from algal cells, algal cultures (50 mL) were transferred into centrifuge tubes and centrifuged at $10,000 \text{ g}$ for 10 min. Separated algal cells were disrupted in liquid nitrogen in a ceramic mortar, then 500 μL of TRIzol reagent (Invitrogen) was added and RNA was extracted according to the manufacturer's instructions (including DNase treatment). Nucleic acid concentrations were measured

spectrophotometrically at 260 nm. The 260/280 nm ratios were determined and used to gage the purity of the total RNA extracted. The integrity of the isolated RNA was tested by electrophoresis on a 1% agarose formaldehyde gel.

Reverse transcription and real-time polymerase chain reaction analysis

To facilitate the real-time polymerase chain reaction (PCR) analysis of the selected genes under the same reaction conditions, primers were first designed by TaKaRa Biochemicals (TaKaRa). Reverse transcription was carried out using an M-MLV reverse transcriptase kit (TaKaRa). Real-time PCR was performed using an Applied Biosystems 7300 (Applied Biosystems). Gene-specific primer pairs of *psaB*, *psbC*, and a housekeeping gene used for PCR were prepared according to a previously reported method (Qian *et al.*, 2009). The 18S rRNA transcript was used to standardize the results by eliminating variations in the quantity and quality of mRNA and cDNA. Each mRNA level was expressed relative to 18S rRNA. A reaction mixture for each PCR run was prepared with the SYBR Green PCR Core Reagents (TaKaRa). The cycle parameters consisted of 1 cycle of 10 s at 95°C and then 40 cycles of 5 s at 95°C followed by 31 s at 60°C. Data were collected at the end of each extension step. Gene expression levels among the treatment groups were analyzed by a previously reported method (Livak and Schmittgen, 2001), where *Ct* is the cycle number at which the fluorescence signal rises statistically above the background.

Data analysis

Data are presented as mean $\pm$ standard error of the mean and were tested for statistical significance using ANOVA followed by the Fisher's *post hoc* test using the StatView 5.0 program (Statistical Analysis Systems Institute). Values were considered significantly different when the probability (*p*) was less than 0.05 or 0.01. For the growth inhibition tests with the green alga, the EC₅₀ values (herbicide concentration required to cause a 50% reduction in growth) were computed. The EC₅₀ values were calculated using linear regression analysis of herbicide concentration (transformed to natural logarithm data) versus percent inhibition (Ma *et al.*, 2004). Subsequent linear regression analysis used SPSS 11.0.

Results

Toxicity of dichloromethane and dichloroethane on the growth of *C. vulgaris*

Effects of dichloromethane and dichloroethane individually and in combination on the growth of *C. vulgaris* are shown in Table 1. The data indicate that dichloromethane and di-

chloroethane are moderately toxic to *C. vulgaris*. The EC₅₀ values were 0.98% and 0.38%, respectively. The dichloromethane and dichloroethane combination was the most acutely toxic on the growth of *C. vulgaris* with an EC₅₀ value of 0.28%.

Effect of dichloromethane and dichloroethane on SOD activity, CAT activity, MDA accumulation, chl *a* expression, and total protein content

Bioassay results (Fig. 1) show clear differences in pigment content (chl *a* and chl *b*) between the control and treatment groups when the algae were exposed to different concentrations of two organic solvents. The cell number, chl *a*, and total protein content gradually decreased with increasing dichloromethane and dichloroethane concentrations over the 96 h of exposure (Figs. 1a–c). Both CAT and SOD activity showed similar patterns in that they increased with increasing dichloromethane and dichloroethane at lower concentrations, while higher doses caused a clear reduction in the antioxidant activity (Figs. 1d, e). The maximum CAT activity was 3.88 times higher compared with the control, and was observed at 96 h of exposure to dichloromethane (Fig. 1e). MDA, an indicator of lipid peroxidation, showed a concentration-dependent increase in response to dichloromethane and dichloroethane exposure (Fig. 1f).

The effects of combined dichloromethane and dichloroethane on *C. vulgaris* cells after 96 h of exposure are shown in Fig. 1. The MDA and CAT levels were virtually unchanged after 96 h of exposure to the dichloromethane and dichloroethane combination EC₅₀ (Figs. 1e, f). The cell number, chl *a*, and total protein content were significantly decreased after 96 h of exposure. In particular, the cell number was 5.06-fold lower compared with the control after 96 h of exposure. However, the SOD activity increased with increasing pollutant concentrations. The above results show the effects were independent of concentrations after 96 h of exposure.

Dichloromethane and dichloroethane effects on subcellular structure

Healthy *C. vulgaris* are smooth plump ellipsoids with clearly visible microfilaments. Morphological abnormalities of *C. vulgaris* cells induced by the pollutants are shown in Fig. 2. The most frequently observed abnormalities were cell shape distortions (Fig. 2d, f), and tissue and cell membrane lysis (Fig. 2h). Other abnormalities were cell shrinkage or swelling (Fig. 2h). The ultrastructure of *C. vulgaris* was compared between the control cells and those exposed to dichloromethane and dichloroethane for 96 h. Compared with the controls (Fig. 3a), TEM analysis showed that many of the chloroplasts were destroyed and the normally highly organized thylakoid

TABLE 1. TOXIC EFFECTS TOWARD GROWTH OF *CHLORELLA VULGARIS*

Pollutant	Regression equation ^a	Correlation coefficient (<i>r</i> ²)	Significance level (<i>P</i>)	EC ₅₀ (mg/L)
Dichloromethane	$P = 1.4145 + 0.2118 \ln C$	0.9618	0.0614	0.98
Dichloroethane	$P = 2.1706 + 0.6012 \ln C$	0.9708	0.0395	0.38
Mixture (v/v, 1:1)	$P = 1.5019 + 0.3195 \ln C$	0.9824	0.0615	0.28

^a*P* and *C* denote percent inhibition and solvent concentration, respectively.
EC, effective concentration.

FIG. 1. Effect of dichloromethane and dichloroethane on superoxide dismutase (SOD) and catalase (CAT) activity, as well as malondialdehyde (MDA), chlorophyll a, and total protein content over the full 96 h of exposure. Algal cell number **(a)**, chlorophyll a **(b)**, total protein content **(c)**, SOD activities **(d)**, CAT activities **(e)**, and MDA content **(f)** in *Chlorella vulgaris* exposed to varying concentrations of pollutant. The y-axis represents cell number and enzyme activity (or the content of MDA), which are expressed as mean $\pm$ standard error of the mean (S.E.M.) of three replicate cultures. * and ** represent statistically significant differences when compared with the control without pollutant exposure at $p < 0.05$ and $p < 0.01$ levels, respectively.

FIG. 2. Micrographs of *Chlorella* cells after exposure to pollutants (EC_{50}) for 96 h: (a, $\times 600$; b, $\times 1500$) control cell; (c, $\times 600$; d, $\times 1500$) dichloromethane-treated cell; (e, $\times 600$; f, $\times 1500$) 1,2-dichloroethane-treated cell; (g, $\times 600$; h, $\times 1500$) in the combination-treated cell. EC, effective concentration.

membranes were damaged. Approximately 13% of the treated cells lost some lamellar structure in the thylakoids (Fig. 3f) and some cells lost the whole chloroplast (Fig. 3e). In some cases, an increased frequency of starch granules was also observed (Fig. 3e). The average numbers of starch granules per cell increased

from approximately two granules in the control to five or six. Occasionally, the treated algae samples were almost devoid of chloroplasts with disrupted lamellar structures (Fig. 3d-f), and the increased starch occupied almost the whole chloroplast (Fig. 3d, e).

FIG. 3. Ultrastructure of *C. vulgaris* cells after exposure to pollutants (EC_{50}) for 96 h: (a) control cell; (b–d) dichloromethane-treated cells; (e, f) 1,2-dichloroethane-treated cells. N, nucleus; Chl, chloroplast; T, thylakoid; CW, cell wall; S, starch granule.

Dichloromethane and dichloroethane effects on photosynthesis-related genes transcription

Figure 4 shows the effects of dichloromethane and dichloroethane at various exposure times on the relative transcript abundance of the *psaB* and *psbC* genes. The transcript abundance of the *psaB* was significantly affected by dichloromethane and dichloroethane (Fig. 4a). After 6–12 h of exposure, the transcript abundance of the *psaB* was significantly higher at the EC_{50} of dichloromethane and dichloroethane compared with the control. The transcript abundance of *psaB* decreased with longer exposure (24 and 64 h). For example, the minimum transcript abundance after 64 h of exposure to dichloroethane was ~59% that of the control sample. Furthermore, *psbC* exhibited distinct responses to dichloromethane and dichloroethane (Fig. 4b). The transcript abundance of *psbC* was significantly decreased after a 6-h exposure to dichloromethane, but the abundance of the *psbC* transcript did not decrease significantly after a 6-h exposure to dichloroethane. In fact, dichloroethane resulted in a 10–30% decrease in *psbC* transcript abundance compared with the control sample. The maximum decrease in *psbC* transcript abundance was observed after 12 h of exposure at the EC_{50} of

dichloromethane and dichloroethane. These results clearly show the strong influence of dichloromethane and dichloroethane on the transcription of photosynthetic genes.

Discussion

Algal cells exposed to contaminants may suffer serious morphological and biochemical alterations (Rocchetta *et al.*, 2006). Dichloromethane and dichloroethane not only disrupt the growth of bacteria and plants, but also show broad toxicity in mammals. Traditionally, the acute toxic effects of organic solvent pollutants on freshwater algae are assessed using single test compounds (Wilde *et al.*, 2006). However, natural pools of water are usually polluted by a mixture of substances that could act synergistically (Shuhaimi-Othman and Pascoe, 2007), and this led us to test the toxic interaction of dichloromethane and dichloroethane.

Dhillon and Von Burg (1995) demonstrated that dichloromethane caused a significant reduction in live phytoplankton cells at 10 mg/L using the flow cytometric technique, while 1 mg/L was not toxic. In our study, low dichloromethane concentrations also had fewer effects on algal growth under laboratory conditions. However, toxicity was

FIG. 4. Effects on transcription of photosynthesis-related genes in *C. vulgaris* cells exposed to the pollutants at EC₅₀. **(a)** Photosystem I reaction center protein subunit B; **(b)** an integral membrane protein component of photosystem II. Values were normalized against 18S rRNA; data represent the relative mean mRNA expression value $\pm$ S.E.M. of three replicate cultures. * and ** represent statistically significant differences when compared with the control without pollutant glufosinate exposure at $p < 0.05$ and $p < 0.01$ levels, respectively.

enhanced in combination with dichloroethane. Moreover, few reports have studied the effects of dichloromethane and dichloroethane on aquatic plants at the physiological or molecular level (i.e., effects short of cell death).

To this end, we analyzed the changes in antioxidant enzymes, ultrastructure, and gene expression in the unicellular green alga *C. vulgaris* in response to dichloromethane and dichloroethane. Similar to other environmental stresses, dichloromethane and dichloroethane causes oxidative damage in algae, both directly and indirectly, by triggering increased levels of reactive oxygen species (ROS). This was also observed during copper toxicity, as well as in response to elevated temperature and UV stress (White and Jahnke, 2002; Andrade *et al.*, 2006). These ROS include the superoxide radical ($O_2^{\cdot-}$), H_2O_2 , and the hydroxyl radical ($\cdot OH$) (Mittler, 2002; Qian *et al.*, 2009). Organisms have a range of antioxidant enzymes and antioxidant substances that protect against the potential damaging effects of these ROS. The activity of one or more of these enzymes is generally increased when plants are exposed to stressful conditions (Artetxe *et al.*, 2002). In this study, dichloromethane and dichloroethane exposure increased the activities of SOD and CAT. SOD is the first step in the removal of ROS by converting $O_2^{\cdot-}$ from electron trans-

port into H_2O_2 and oxygen. The increase in the activity of SOD in response to dichloromethane and dichloroethane suggests a possible stress-induced induction of the SOD enzyme. Similarly, the increased activity of CAT indicates a stress-induced upregulation in response to increased H_2O_2 accumulation. Indeed, CAT and SOD upregulation may have protected algae against solvent toxicity, at least at low solvent concentrations. The consistently high levels of MDA, however, suggest that the antioxidant enzymes induced by dichloromethane and dichloroethane were not be able to eliminate ROS sufficiently to protect cells from damage.

Chloroplasts contain a highly organized thylakoid membrane system and provide all structural properties for optimal light harvesting (Allen and Forsberg, 2001). The destruction of chloroplasts can disrupt normal photosynthesis. Our TEM analysis showed that dichloromethane and dichloroethane increased the number of starch granules. An increase in starch content was also observed by Morlon *et al.* (2005), who showed that starch granules were overproduced by algae in response to selenite exposure. TEM also showed that dichloromethane and dichloroethane partially damaged the structure of chloroplasts, and this could be observed visually, as cells turned from green to white when exposed to

dichloromethane and dichloroethane observed in this study. The structural damage to the chloroplasts induced by dichloromethane and dichloroethane was likely accompanied by a reduction in the photosynthetic activity. Coetzer and Al-Khatib (2001) described a rapid reduction in the photosynthetic rate 2 h after pesticide application. Our result demonstrated that exposure to dichloromethane and dichloroethane resulted in changes in the transcript abundance of the photosynthesis-related genes *psaB* and *psbC*. Compared with the controls, the transcript abundance of *psaB* decreased significantly after exposure to EC₅₀ concentrations of dichloromethane and dichloroethane, whereas the abundance of *psbC* increased after exposure to EC₅₀ dichloroethane. The decrease in transcript abundance resulted in a decrease in the amount of encoded enzyme and its activity. Rubisco has both carboxylase and oxygenase activity that controls the rate-limiting step in carbon assimilation and photorespiration, respectively. Photorespiration serves as the electron sink for the consumption of excess reducing equivalents (Asada, 1994). In addition, the photorespiratory pathway plays an important role in the prevention of photoinhibition. On the other hand, carboxylase can also use reducing equivalents to fix CO₂ (e.g., ATP and NADPH). By decreasing the abundance of *psaB* and blocking carbon assimilation and photorespiration, hydrocarbons cause the accumulation of a mass of reducing equivalents. Excess electrons can lead to decreased transcription of PSI and PSII genes. This, in turn, reduces electron flow through PSI and PSII. In this study, we observed a decrease in the expression of photosynthesis-related genes, *psbC* and *psaB*. Electron transport initially occurs in PSII and is then relayed to PSI. The inhibition of electron transport first occurred in PSII because the transcript abundance of *psaB* decreased at 6 h of exposure, whereas the abundance of *psbC* increased. When electron transport is blocked at PSII, algae tend to increase the expression of related proteins at PSI to enhance electron receptivity from PSII. This strategy may enable photosynthesis to proceed as normally as possible, especially under adverse conditions (Pfannschmidt, 2003). When the environmental pressures (e.g., continuous organic solvent exposure in the present study) exceed the organism's ability to tolerate the stress, normal metabolism is inevitably disrupted, resulting in a subsequent decrease in the transcript abundance of *psaB*.

Until recently, chloroplast genes were erroneously believed to be regulated at the post-transcriptional level (Pfannschmidt, 2003). In fact, gene expression involves several steps starting with the transcription of a gene or operon into a pre-mRNA. Various processing steps, including splicing and editing, result in a mature mRNA molecule. The mRNA must be finally loaded onto polyribosomes to obtain a functional polypeptide. Photosynthetic gene expression is possibly regulated at most of these steps, as reported in several recent studies (Golden, 1995; Link, 1996; Allison, 2000).

The search for novel biomarkers for environmental risk assessment and pollution monitoring has become a hot topic during the last few decades (Lagadic *et al.*, 2000). In aquatic ecosystems, algae are primary producers, providing oxygen and organic substances to other life forms. In recent years, algae have been widely used in ecological risk assessment to evaluate the impacts of metal, herbicide, and other xenobiotic contamination and bioavailability in aquatic systems, since they are sensitive to hydrocarbon contaminants at environ-

mentally relevant concentrations. Molecular biomarker development has focused on several toxic chemical targets, such as transcription levels of photosynthesis-related genes and antioxidant enzymes for herbicide monitoring. However, many of the classic biomarkers used in ecotoxicology are nonspecific, for they are commonly employed in monitoring general pollutants. Most of the knowledge on the toxicity of xenobiotic pollutants to freshwater algae is based upon the effects of compounds tested in the laboratory by evaluating biochemical parameters such as EC₅₀, chlorophyll levels, biovolume, cell count, and cell size. The results will be proposed to be potential biomarkers for the evaluation and monitoring of organic solvent contamination in the aquatic ecosystems.

Conclusion

Results from this study suggest that dichloromethane and dichloroethane alter gene transcription, cell ultrastructure, and the metabolic state of *C. vulgaris*. The inhibitory and stimulatory effects of organic solvents depend on concentrations. Different photosynthetic genes, however, have different sensitivities to the same toxicant and the same target sites may be more or less damaged by different toxicants. To our knowledge, few studies have reported the effects of dichloromethane and dichloroethane on an aquatic organism such as *C. vulgaris*. Dichloromethane and dichloroethane not only change the antioxidant enzyme activity, MDA levels, and ultrastructure of this aquatic organism, but also affect the expression of photosynthetic genes at the transcriptional level.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Grant No. 20977087) and the Natural Science Foundation of Zhejiang Province, China (Grant No. LY13B070008). We would also like to thank Dr. Haifeng Qian and Prof. Weihong Zhong for the helpful discussions and the good working conditions.

Author Disclosure Statement

No competing financial interests exist.

References

- Alexander, V.P., and Christine, C.W. (2000). A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clin. Chim. Acta* 293, 157.
- Allen, J.F., and Forsberg, J. (2001). Molecular recognition in thylakoid structure and function. *Trends Plant Sci.* 6, 317.
- Allison, L.A. (2000). The role of sigma factors in plastid transcription. *Biochimie* 82, 537.
- Andrade, S., Contrerasa, L., Moffettb, J.W., and Correea, J.A. (2006). Kinetics of copper accumulation in *Lessonia nigrescens* (Phaeophyceae) under conditions of environmental oxidative stress. *Aquat. Toxicol.* 78, 398.
- Artetxe, U., Garcia-Plazaola, J.I., Hernandez, A., and Becerril, J.M. (2002). Low light grown duckweed plants are more protected against the toxicity induced by Zn and Cd. *Plant Physiol. Biochem.* 40, 859.
- Asada, K. (1994). Production and action of active oxygen species in photosynthetic tissues. In: C.H. Foyer and P.M. Mullineaux,

- Eds., *Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants*. Boca Raton, FL: CRC Press, pp. 77–104.
- Coetzer, E., and Al-Khatib, K. (2001). Photosynthetic inhibition and ammonium accumulation in Palmer amaranth after glufosinate application. *Weed Sci.* 49, 454.
- Dhillon, S., and Von Burg, R. (1995). Toxicology update. Methylene chloride. *J. Appl. Toxicol.* 15, 329.
- Fathi, A.A., El-Shahed, A.M., and Shoukamy, M.A. (2008). Response of Nile water phytoplankton to the toxicity Cobalt, Copper and Zinc. *Res. J. Environ. Toxicol.* 2, 67.
- Glenn, W.S., and Tara, M.S. (1988). Interaction of organic solvents with the green alga *Chlorella pyrenoidosa*. *Bull. Environ. Contam. Toxicol.* 40, 736.
- Golden, S.S. (1995). Light-responsive gene expression in cyanobacteria. *J. Bacteriol.* 177, 1651.
- Inskip, W.P., and Bloom, P.R. (1985). Extinction coefficients of chlorophyll a and b in N,N-dimethylformamide and 80% acetone. *Plant Physiol.* 77, 483.
- Lagadic, L., Caquet, T., Amiard, J.C., and Francois, R. (2000). *Use of Biomarkers for Environmental Quality Assessment*. Rotterdam: A.A. Balkema.
- Levy, J.L., Stauber, J.L., and Jolley, D.F. (2007). Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity. *Sci. Total Environ.* 387, 141.
- Link, G. (1996). Green life: Control of chloroplast gene transcription. *BioEssays* 18, 465.
- Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR method. *Methods* 25, 402.
- Ma, J., and Liang, W. (2001). Acute toxicity of 12 herbicides to the green alga *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. *Bull. Environ. Contam. Toxicol.* 67, 347.
- Ma, J., Lin, F., Wang, S., and Xu, L. (2004). Acute toxicity assessment of 20 herbicides to the green alga *Scenedesmus quadricauda* (Turp.) Breb. *Bull. Environ. Contam. Toxicol.* 72, 1164.
- Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405.
- Morlon, H., Fortin, C., Floriani, M., Adam, C., Garnier-Laplace, J., and Boudou, A. (2005). Toxicity of selenite in the unicellular green alga *Chlamydomonas reinhardtii*: Comparison between effects at the population and subcellular level. *Aquat. Toxicol.* 7, 65.
- Nriagu, J.O., and Pacyna, J.M. (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature* 333, 134.
- Penuelas, J., and Filella, I. (2002). Metal pollution in Spanish terrestrial ecosystems during the twentieth century. *Chemosphere* 46, 501.
- Pfannschmidt, T. (2003). Chloroplast redox signals: How photosynthesis controls its own genes. *Trends Plant Sci.* 8, 33.
- Piero, F., Lorenzo, P., and Giovanni, B. (1980). Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymatic assay of uric acid in serum and urine. *Clin. Chem.* 26, 227.
- Qian, H.F., Chen, W., Sheng, G.D., Xu, X.Y., Liu, W.P., and Fu, Z.W. (2008a). Effects of glufosinate on antioxidant enzymes, subcellular structure, and gene expression in the unicellular green alga *Chlorella vulgaris*. *Aquat. Toxicol.* 88, 301.
- Qian, H.F., Sheng, G.D., Liu, W.P., Lu, Y.C., Liu, Z.G., and Fu, Z.W. (2008b). Inhibitory effects of atrazine on *Chlorella vulgaris* as assessed by real-time polymerase chain reaction. *Environ. Toxicol. Chem.* 27, 182.
- Qian, H.F., Xu, X.Y., Chen, W., Jiang, H., Jin, Y.X., Liu, W.P., and Fu, Z.W. (2009). Allelochemical stress causes oxidative damage and inhibition of photosynthesis in *Chlorella vulgaris*. *Chemosphere* 75, 368.
- Rocchetta, I., Mazzuc, V., and Carmen, M.R. (2006). Effect of chromium on the fatty acid composition of two strains of *Euglena gracilis*. *Environ. Pollut.* 141: 353.
- Shuhaimi-Othman, M., and Pascoe, D. (2007). Bioconcentration and depuration of copper, cadmium, and zinc mixtures by the freshwater amphipod *Hyalella azteca*. *Ecotoxicol. Environ. Saf.* 66, 29.
- Stauber, J.L., and Davies, C.M. (2000). Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. *Environ. Rev.* 8, 255.
- Trotsenko, Y.A., Doronina, N.V., Tourova, T.P., Kuznetsov, B.B., and Leisinger, T. (2000). *Methylophila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov.—Novel aerobic facultatively methylotrophic bacteria utilizing dichloromethane. *Syst. Appl. Microbiol.* 23, 210.
- White, A.L., and Jahnke, L.S. (2002). Contrasting effects of UV-A and UV-B on photosynthesis and photoprotection of β -carotene in two *Dunaliella* spp. *Plant Cell Physiol.* 43, 877.
- Wilde, K.L., Stauber, J.L., Markich, S.J., Franklin, N.M., and Brown, P.L. (2006). The effect of pH on the uptake and toxicity of copper and zinc in a tropical freshwater alga (*Chlorella* sp.). *Arch. Environ. Contam. Toxicol.* 51, 174.
- Zhang, J.X., and Kirkham, M.B. (1994). Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. *Plant Cell Physiol.* 35, 785.
- Zhou, Y., and Zhang, Z. (1989). *The Toxicity Methods in Aquatic Living*. Beijing, Agriculture Press.