ELSEVIER

Aquatic Toxicology 85 (2007) 192–201

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Toxicity and metabolism of *p*-chlorophenol in the marine microalga *Tetraselmis marina*

Dimitris Petroutsos [a,b], Jiangxin Wang [a], Petros Katapodis [b], Dimitris Kekos [b], Milton Sommerfeld [a], Qiang Hu [a,*]

[a] *Department of Applied Biological Sciences, Arizona State University Polytechnic Campus, 7001 E. Williams Field Road, Mesa, AZ 85212, United States*

[b] *Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Street, Zografou Campus, Athens 15780, Greece*

Received 14 July 2007; received in revised form 4 September 2007; accepted 5 September 2007

Abstract

Toxicity and metabolism of *para*-chlorophenol (*p*-CP) in the marine microalga *Tetraselmis marina* have been studied. The inhibition constant EC_{50} for *p*-CP was $272 \pm 17\,\mu M$ ($34.8 \pm 2.2\,mg\,L^{-1}$) under the experimental conditions. Two metabolites were detected in the growth medium in the presence of *p*-CP by reverse phase HPLC and their concentrations increased at the expense of *p*-CP. The two metabolites, which were found to be more polar than *p*-CP, were isolated by a C18 column. They were identified as *p*-chlorophenyl-β-D-glucopyranoside (*p*-CPG) and *p*-chlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (*p*-CPGM) by electrospray ionization-mass spectrometric analysis in a negative ion mode. The molecular structures of *p*-CPG and *p*-CPGM were further confirmed by enzymatic and alkaline hydrolyses. Treatment with β-glucosidase released free *p*-CP and glucose from *p*-CPG, whereas *p*-CPGM was completely resistant. Alkaline hydrolysis completely cleaved the esteric bond of the malonylated glucoconjugate and yielded *p*-CPG and malonic acid. It was concluded that the pathway of *p*-CP metabolism in *T. marina* involves an initial conjugation of *p*-CP to glucose to form *p*-chlorophenyl-β-D-glucopyranoside, followed by acylation of the glucoconjugate to form *p*-chlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside. The metabolism of *p*-CP in *T. marina* was mainly driven by photosynthesis, and to a lesser extent by anabolic metabolism in the dark. Accordingly, the detoxification rate under light was about seven times higher than in the darkness. This work provides the first evidence that microalgae can adopt a combined glucosyl transfer and malonyl transfer process as a survival strategy for detoxification of such xenobiotics as *p*-CP.

Keywords: *Tetraselmis marina*; Marine microalgae; Chlorophenol metabolism; Toxicity; Biotransformation

1. Introduction

Chlorinated phenols and their degradation byproducts have been found in many fresh and marine waters, mainly due to anthropogenic inputs from industrial wastes and degradation of chlorinated pesticides (WHO, 1989; Lee et al., 1991; Kot-Wasik et al., 2004; Dimou et al., 2006). The pollution of coastal sea waters with chlorinated phenols are of environmental concern because these compounds can be toxic to organisms of different trophic levels in the food chain and are recalcitrant to treatment, whereas some of the chlorophenols may bioaccumulate in aquatic organisms (Renberg et al., 1980; Kuiper and Hanstveit, 1984; Fleming, 1995; Araki et al., 2000).

Chlorophenol toxicity to aquatic organisms has been shown to increase with the degree of chlorination of the phenol ring, presumably as a result of increasing lipophilicity (WHO, 1989). Depending on the species of organisms and specific chlorophenols tested, the LC_{50} value may range from 0.002 to 9.8 mg/L (Webb and Brett, 1973; Van Dyk et al., 1977; Borthwich and Schimmel, 1978; Mcleese et al., 1979; Hauch et al., 1980; USEPA, 1980; Hattula et al., 1981; Kuiper and Hanstveit, 1984). The United States Environmental Protection Agency (USEPA) has set a maximum contaminant level for chlorophenolic compounds at $1\,\mu g\,L^{-1}$ or less (Jennings et al., 1996).

Some higher plants have been shown to be able to metabolize a broad range of xenobiotics, whereby phenolic compounds

* Corresponding author. Tel.: +1 480 727 1484; fax: +1 480 727 1236.
E-mail address: huqiang@asu.edu (Q. Hu).

0166-445X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2007.09.007

are converted to β-*O*-glucosides by glucosyltransferases in the presence of uridine diphosphate (UDP) glucose as the glucosyl donor. The glucosylated phenols may undergo further metabolism to form 6-*O*-malonate hemi-ester glucose conjugates, which are common forms of complex glucoside conjugates produced in higher plants. These glucoside conjugates are formed by the action of malonyl-CoA transferase on glucose conjugates in the presence of malonyl CoA (Lamoureux and Rusness, 1986). Glucosidation of chlorophenols and/or subsequent malonylation have been previously described in terrestrial angiosperms with soybean, wheat, and cotton being examples (Schmitt et al., 1985; Laurent et al., 2000; Pascal-Lorbel et al., 2003), but reported in the aquatic angiosperms *Lemna gibba* and *Lemna minor* as well (Sharma et al., 1997; Day and Saunders, 2004).

Pridham (1964) reported that within 10 species of macroalgae (in both freshwater and marine forms) no glucosylation of the phenolic compounds quinol or resorcinol was observed. Later Pflugmacher and Sandermann (1998) showed that several marine macroalgae had *O*-glucosyltransferase activity with respect to chlorophenols. Given the widespread distribution and ecological importance of marine microalgae as primary producers and base of the food chain it is important to understand possible toxicity and metabolism of chlorophenols in marine microalgae.

Data concerning toxicity and metabolism of chlorophenols in marine microalgae is limited to two species of diatoms. *Thalassiosira* sp., for instance, degraded phenol with simultaneous production of both protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase (*ortho*- and *meta*-pathway aromatic ring cleavage enzymes) activities. Cell-free extracts of this diatom previously grown on phenol were capable of dechlorinating monochlorophenols, 3,5-dichlorophenol, and 2,4,6-trichlorophenol (Lovell et al., 2002). In a separate study, Yang et al. (2002) observed that the addition of glutathione to the diatom *Skeletonema costatum* culture enhanced metabolism of 2,4-dichlorophenol with little increase in biomass, suggesting that the molecular mechanism involved in the metabolism of 2,4-dichlorophenol in this organism was glutathione conjugation.

Tetraselmis marina, a common coastal prasinophyte, has been shown to grow in the presence of and remove monosubstituted chlorophenols from growth medium, with high efficiency to *p*-chlorophenol. In closed static flasks containing an initial concentration of 1 g L^{-1} $NaHCO_3$ and under continuous illumination *T. marina* removed 102 μM *p*-chlorophenol over a 10 day cultivation period (Petroutsos et al., 2007).

The aim of the present study was to elucidate the metabolic pathway of *p*-chlorophenol in *T. marina* and compare it with metabolic pathway(s) of this chemical in other algae and higher plants.

2. Materials and methods

2.1. Organism and culture conditions

An axenic culture of *T. marina* CCMP 898 was obtained from the Provasoli–Guillard National Centre for the Culture of Marine Phytoplankton, Maine, USA (Collection site: North Pacific, 48.2200N 122.7700W Partridge Point, Whidbey Island, Washington, USA). The microalga was maintained in sterile artificial reconstituted seawater (Natural Sea Salt Mix, Oceanic Products, Franklin, WI, USA) at 34 ppt, enriched with Guillard's f/2 nutrient medium (Guillard, 1975) in 250 mL Erlenmeyer flasks closed with cotton plugs, without shaking. Flasks were illuminated at 40 μE m^{-2} s^{-1} by cool-white fluorescent light at 20 °C. Stock cultures were transferred to fresh medium every 7 days.

2.2. Chemicals

p-Chlorophenol was obtained from Sigma Chemical Ltd. (St. Louis, MO, USA). Methanol HPLC grade was obtained from Fisher Scientific (Houston, TX, USA). All other chemicals used in this study were from Sigma Chemical Ltd. and were reagent grade or higher purity.

2.3. Experimental system

All experiments were carried out in glass columns (2.6 cm i.d., 45 cm length, 250 mL culture capacity) at room temperature (20 °C). Mixing of cultures was provided by bubbling CO_2 enriched compressed air (1% v/v) through a capillary tube (i.d., 1 mm) that opened near the bottom of the column (Hu et al., 2000) at a rate of 0.6 volume of air per volume of culture per minute (vvm). Light was provided by a panel of fluorescent lamps on one side of the columns and light intensity was 60 μE m^{-2} s^{-1}. Cultures at a mid-exponential growth phase were used as inocula for experiments. *p*-CP was dissolved in pure methanol, 500 μL of which were added to the cultures in order to obtain final concentrations from 80 to 625 μM. Cultures spiked with 500 μL of pure methanol served as controls. Columns containing *p*-CP dissolved in the culture medium without algal cells were used as blanks.

2.4. Algal growth measurement

Cell density was monitored as optical density at 750 nm (OD_{750}) of the algal suspension in a 1 cm cuvette using a Beckman DU 640 Spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). Cell dry weight (DCW) was measured using 10 mL algal suspension filtered through a preweighed, 0.45 μm filter (Pall Corp., New York, USA) and then washed with 20 mL of 0.5 M ammonium formate. The filters were then dried at 70 °C to a constant weight, and cooled to the room temperature in a vacuum desiccator before weighed (Zhu and Lee, 1997).

Microalgal growth curves under different initial *p*-CP concentrations were plotted against time, and the area under each growth curve was calculated according to the following Eq. (1) (OECD, 1984).

$$A = \left[\frac{N_1 - N_0}{2}\right] t_1 + \left[\left(\frac{N_1 + N_2 - 2N_0}{2}\right)\right] (t_2 - t_1) + \cdots + \left[\left(\frac{N_{n-1} + N_n - 2N_0}{2}\right)\right] (t_n - t_{n-1}) \quad (1)$$

where A was area, N_0 was nominal number of cells mL^{-1} at time t_0, N_1 was measured number of cells mL^{-1} at t_1, N_n was measured number of cells mL^{-1} at time t_n, t_1 was time of first measurement after beginning of test, and t_n was time of nth measurement after beginning of test. The percentage inhibition of the cell growth at each concentration (I_A) was calculated as the difference between the area under the control growth curve (A_c) and the area under the growth curve at each test substance concentration (A_t) as shown in Eq. (2):

$$I_A = \left(\frac{A_c - A_t}{A_c}\right) \times 100 \quad (2)$$

The data were fitted with the following four parameter logistic regression model (Hill equation) to determine EC_{50} values with the use of Sigma plot 9.01 (Systat software Inc., Richmond, USA).

$$y = \min + \frac{\max - \min}{(1 + x/EC_{50})^{\text{Hillslope}}} \quad (3)$$

where y denotes the response (percentage inhibition of the microalgal growth at each p-CP concentration, as calculated from Eq. (2)); min and max are the levels of response at zero and infinite p-CP concentration respectively, Hillslope is a slope variable denoting the steepness of the dose–response curve, EC_{50} is p-CP concentration that produces the response halfway between the max and min response levels and x is p-CP concentration (Motulsky and Christopoulos, 2003).

2.5. Analytical methods

For p-chlorophenol analysis *T. marina* cells were separated from the culture broth by centrifugation at 12,000 × g for 5 min. The supernatant was filtered through a 0.45 μm nylon filter (Fisher Scientific, Houston, TX, USA) and the filtrate was further used as the source of p-CP. Analysis was performed by HPLC using a 5 μm, 4.6 mm × 250 mm Spherisorb ODS2 column (Waters, Milford, MA, USA). Detection was made by a 2996 Waters photodiode array detector set at 280 nm using a 2695 Waters separation module. The isocratic solvent system used was 49/50/1 (v/v/v) water/methanol/acetic acid at a flow rate of 1.0 mL min^{-1} and at 25 °C. Malonic acid was detected at 220 nm using the same column, with a mobile phase of 10 mM KH_2PO_4–CH_3OH (95:5, pH 2.7) at a flow rate of 0.8 mL min^{-1} (Ding et al., 2006).

Enzymatic hydrolysates were analyzed for neutral monosaccharide content by high-performance anion exchange chromatography (HPAEC) using a 4.0 mm × 250 mm CarboPac PA1 column (Dionex, Sunnyvale, CA, USA), a Dionex ED-40 electrochemical detector (gold working electrode and pH reference electrode), and a Waters 600E separation module. The mobile phase consisted of isocratic 20 mM NaOH at a flow rate of 1 mL min^{-1}.

2.6. Metabolites identification

In order to facilitate identification, metabolites were concentrated using a solid phase extraction (SPE) method. A 10 mL aliquot of culture centrifugate was loaded onto a Supelclean LC18 SPE tube (Supelco, Bellefonte, PA, USA) containing 1 g C18. The tube was first conditioned by eluting 2 mL methanol and then 2 mL artificial seawater. The p-CP metabolites were retained and the tube was eluted with 10 mL water. The remaining target compounds were then eluted from tubes with methanol. Methanol fractions (1 mL) were collected in microcentrifuge tubes and analyzed by HPLC. The metabolites were separately collected at the diode array detector cell outlet, in a methanol/water/acetic acid (50/49/1) mixture for mass spectrometric analysis.

Each analyte was directly infused into a Perkin-Elmer Sciex (Thornhill, Canada) API 365 triple quadrupole mass spectrometer (MS) equipped with a turbo ion spray interface, at a flow rate of 1 μL min^{-1}. The total run time was 5 min. The MS was operated in a scan mode from *m/z* 100 to *m/z* 1000. The optimized conditions of MS with the turbo ion spray source were as follows: 9 psi nebulizer gas pressure, 7 psi curtain gas pressure, 2 L min^{-1} turbo gas flow, −4200 V ionspray voltage, −35 V declustering potential, −200 V focusing potential and −10.7 V entrance potential. For the precursor ion scan the triple quadrupole MS was run at the unit-resolution of Q1 and the low-resolution of Q3 in a negative mode. The MS was interfaced to a computer workstation running Analyst software for data acquisition and processing (Version 1.3; Applied Biosystems, Foster City, CA).

2.7. Enzymatic hydrolysis

Enzymatic hydrolysis was accomplished with β-glucosidase from almonds (3 mg mL^{-1}, 6 units mg^{-1} specific activity, Fluka) at 25 °C and pH 5.0 (40 mM phosphate–citrate buffer) for 8 h. β-Glucosidase was first desalted using a PD 10 column (G-25 Sephadex, Pharmacia). The products of the reaction were analyzed by HPLC and HPAEC as described above.

2.8. Alkaline hydrolysis

Samples were incubated with 0.1 N NaOH for 12 h at room temperature. After hydrolysis, alkaline samples were acidified to pH 3.0 with HCl and analyzed by HPLC.

2.9. Light microscopy

T. marina cells were imaged using an Olympus BH-2 microscope (Olympus Optical Co, Ltd., Tokyo, Japan) with a 100× phase contrast objective connected to a Optronics camera (Optronics, Galeta, CA) and compiled with PictureFrame software (Optronics, Galeta, CA).

3. Results

3.1. Effect of initial p-CP concentration on growth and cell aggregation of T. marina

The time-course of growth kinetics of *T. marina* at various initial p-CP levels is presented in Fig. 1. The cells grew in the presence of p-CP at a concentration up to 160 μM with little or

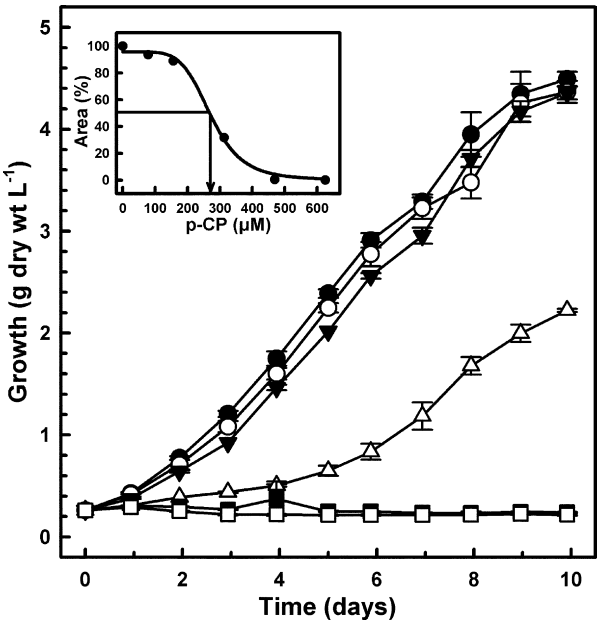

Fig. 1. Photoautotrophic growth of *T. marina* in the absence (●) and presence of initial 80 (◯), 160 (▼), 310 (△), 470(■) and 625 (□) μM *p*-CP. The inset figure shows the dose–response curve representing growth inhibition of the microalga (as % area under the growth curve). The light intensity was 60 μE m^{-2} s^{-1}, the culture temperature was 20 °C, and the aeration rate was ca. 0.6 vvm (error bars indicate standard deviations; $n = 3$).

slight reduction in the specific growth rate compared to the control culture. Significant reduction in growth occurred in cultures spiked with 310 μM *p*-CP and growth was completely inhibited at 470 μM *p*-CP or higher. The inhibition constant EC_{50} for *p*-CP was estimated to be 272 ± 17 μM using Eqs. (1)–(3) described in Section 2.4.

p-CP affected not only growth but also cell aggregation. While the cells remained in a vegetative flagellate stage in the absence of *p*-CP or when the initial *p*-CP concentration was below 160 μM, considerable cell aggregation occurred in cultures spiked with ≥310 μM *p*-CP, and yet the higher the initial *p*-CP concentration, the earlier cell aggregation occurred (Fig. 2). Aggregation of *Tetraselmis* cells were previously observed in cultures containing 10 μg ml^{-1} of marine invertebrate lectin SLL-2 (Koike et al., 2004), or high concentrations of cadmium (da Costa and de França, 1998).

3.2. Uptake and metabolism of p-CP by T. marina

Growth of *T. marina* was coupled with uptake of *p*-CP from the culture medium, and the higher the initial concentration of *p*-CP the higher the uptake rate. Accordingly, the rate of *p*-CP uptake by *T. marina* cells was increased from −0.52 ± 0.04 to −1.27 ± 0.09 to −1.71 ± 0.04 μmol *p*-CP h^{-1} g dry wt^{-1} as the initial concentration of *p*-CP was increased from 80 to 160 to 310 μM. No *p*-CP uptake was observed at initial *p*-CP concentrations higher than 310 μM.

During growth of *T. marina* in the presence of *p*-CP, two metabolites, named P1 and P2, were formed and accumulated in the growth medium (Fig. 3). The two metabolites were more polar than *p*-CP, and their appearances were concomitant with the disappearance of *p*-CP.

3.3. Identification of metabolites

Negative ESI mass spectra of P1 and P2 are shown in Fig. 4A and 4B. The presence of chlorine signatures in all prominent peaks indicated the presence of the parent compound (*p*-CP,

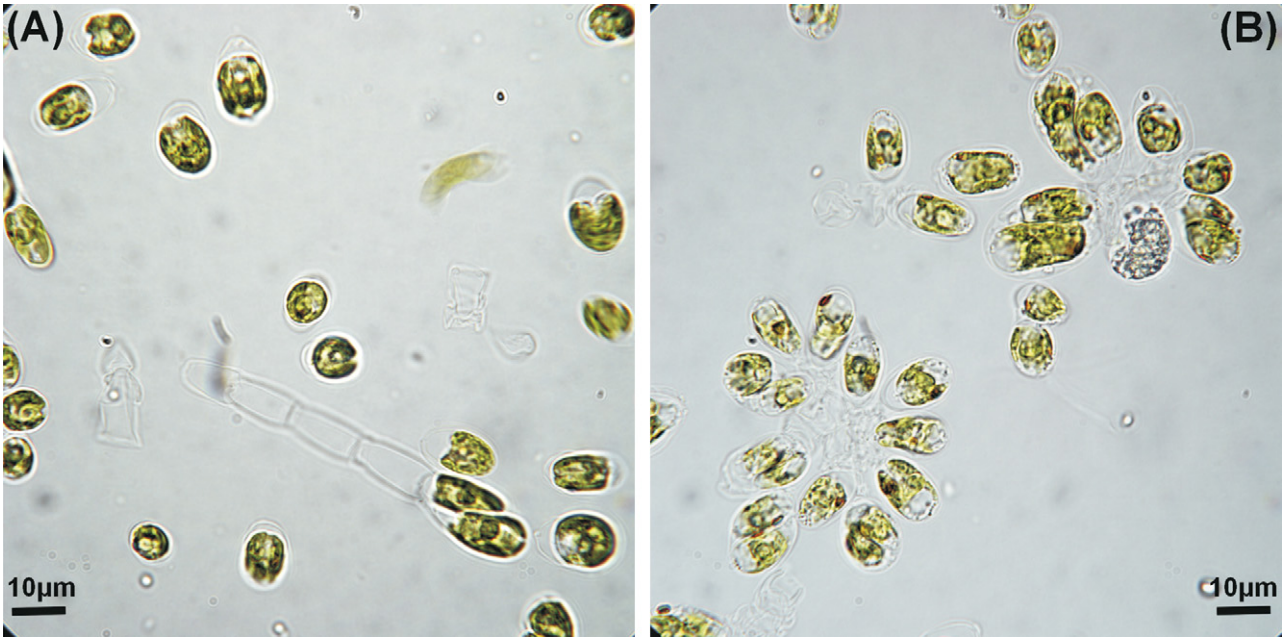

Fig. 2. Light micrographs of *T. marina* cells in control and *p*-CP treated cultures. (A) *T. marina* cells in control cultures most of them either in the flagellate stage, as actively swimming cells, or in the vegetative stage. A blurred actively swimming cell in rapid motion and a septate stalked cell are shown. (B) After 48 h of exposure to 312 μM *p*-CP, cells appeared clamped and aggregated.

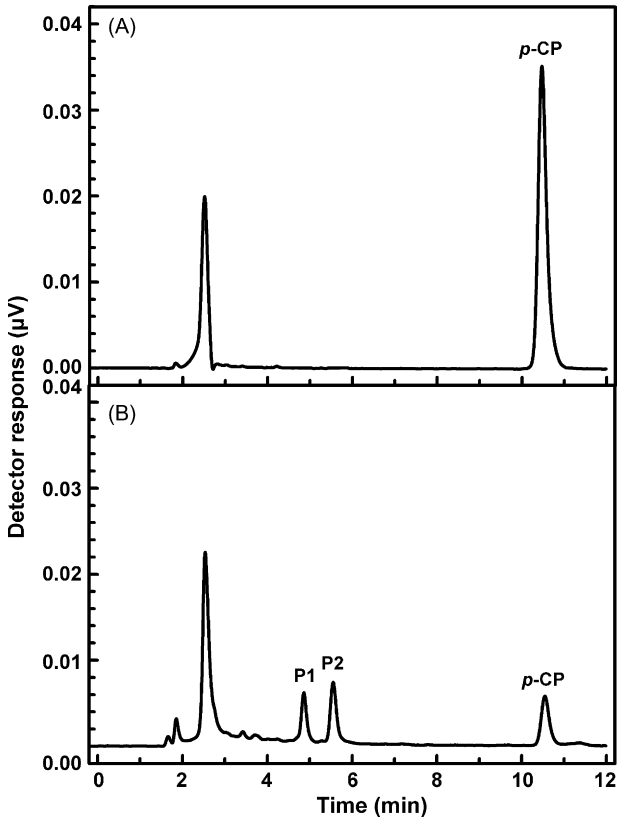

Fig. 3. HPLC profile of *p*-CP and its metabolites P1 and P2, obtained from the centrifugate of *p*-CP treated *T. marina* cells at $t = 0$ (A) and $t = 5$ days (B).

m/z 127) in the metabolites P1 and P2. The analysis of the two metabolites was extended by conducting precursor ion scan of *m/z* 127 (Fig. 5A and 5B). For each peak the degree of chlorination was determined by comparison of percent relative abundance of $m+2$ intensities with the theoretical values based on the natural abundance of $^{35}Cl/^{37}Cl$.

For P1, the *m/z* 579 peak with two chlorine atoms was considered to be a dimer of *m/z* 289 (one chlorine atom), whereas the *m/z* peak 349 was assigned to be a chlorophenol derivative containing a chlorine atom and an acetate ion adduct. A nominal molecular weight of 290 was assigned to the metabolite P1.

For P2 the *m/z* 751 with two chlorine atoms was the dimer of *m/z* 375. The observed *m/z* 44 loss, *m/z* 375–331, was attributed to CO_2 loss and was consistent with primary fragmentation of negative ions produced by ESI of dicarboxylic acids and dicarboxylic acid esters. Malonic acid decarboxylation yielded carbon dioxide and the enolate anion of acetic acid (Grossert et al., 2005). The nominal weight assigned to P2 was 376.

The nominal weights assigned to P1 and P2 were analogous (one chlorine difference) with the nominal weights that Day and Saunders (2004) assigned to 2,4-dichlorophenyl-β-D-glucopyranoside and 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside, after comparison of their ESI-MS spectra with those of synthesized compounds. These compounds were formed during metabolism of 2,4-dichlorophenol by the freshwater macrophyte *Lemna minor*. Based on this, we suggest that P1 was *p*-chlorophenyl-β-D-glucopyranoside (*p*-CPG) and P2 was *p*-chlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (*p*-CPGM).

p-CPG and *p*-CPGM structures were further confirmed by enzymatic and alkaline hydrolyses. Incubation of P1 (*p*-CPG) fraction with β-glucosidase released free *p*-CP, while *p*-CPGM was completely resistant to the β-glucosidase treatment (Fig. 6). The release of free glucose from the enzymatic hydrolysis was confirmed by HPAEC.

Alkaline hydrolysis of P2 (*p*-CPGM) fraction resulted in complete cleavage of the esteric bond of the malonylated glucoconjugate (*p*-CPGM) to release *p*-CPG (Fig. 7). The release of malonic acid as a byproduct of the alkaline hydrolysis was confirmed by HPLC analysis.

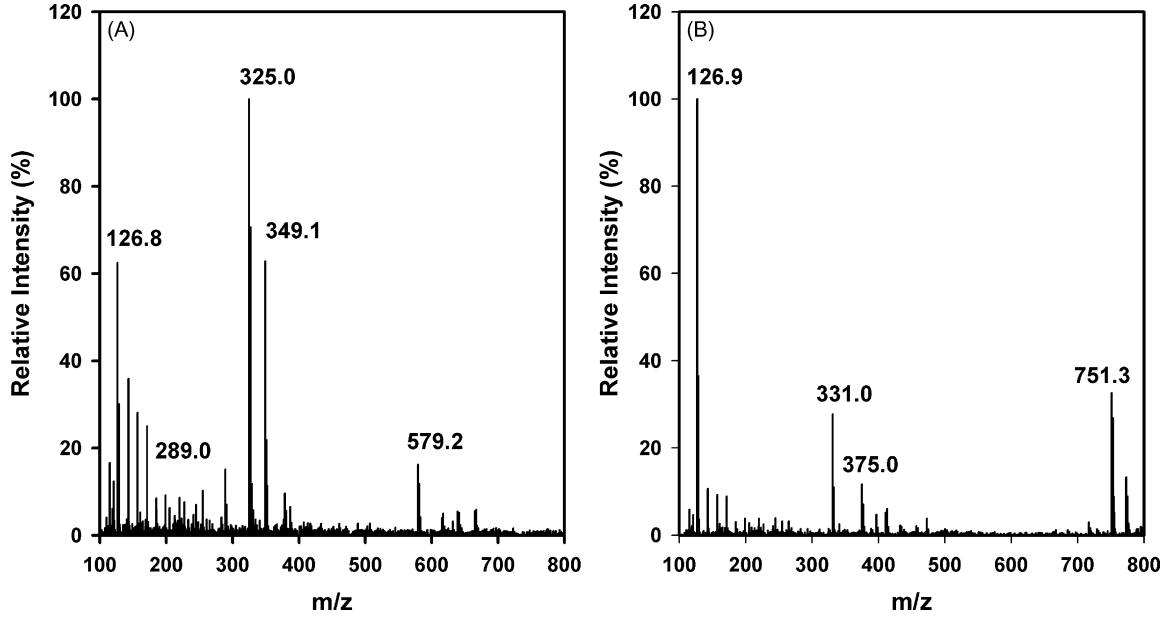

Fig. 4. Full scan mass spectra, obtained by negative ESI-MS, of metabolites P1 (A) and P2 (B), isolated from the culture medium of *p*-CP treated *T. marina* cells.

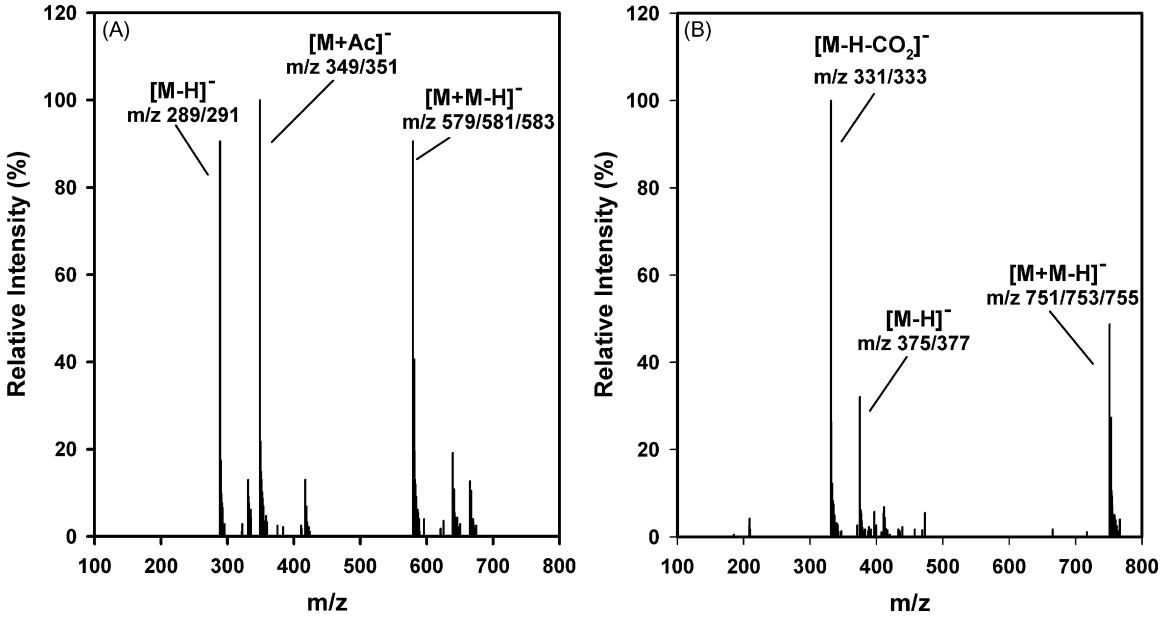

Fig. 5. Precursor ion scan of *m/z* 127 for P1 (A) and P2 (B) metabolites, isolated from the culture medium of *p*-CP treated *T. marina* cells.

The time course of *p*-CPG and *p*-CPGM formation at the expense of *p*-CP during the cultivation of *T. marina* is shown in Fig. 8. *p*-CPG and *p*-CPGM were quantified on the basis of *p*-CP release after enzymatic and alkaline hydrolyses. Less than 10% of *p*-CP removal was due to abiotic loss. The two metabolites formed accounted for ca. 85% of *p*-CP introduced into the culture. It was concluded that the major pathway of *p*-CP metabolism in *T. marina* involves an initial enzymatic synthesis of a *p*-chlorophenyl-β-D-glucopyranoside conjugate (*p*-CPG), followed by acylation to form *p*-chlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (*p*-CPGM) (Fig. 9).

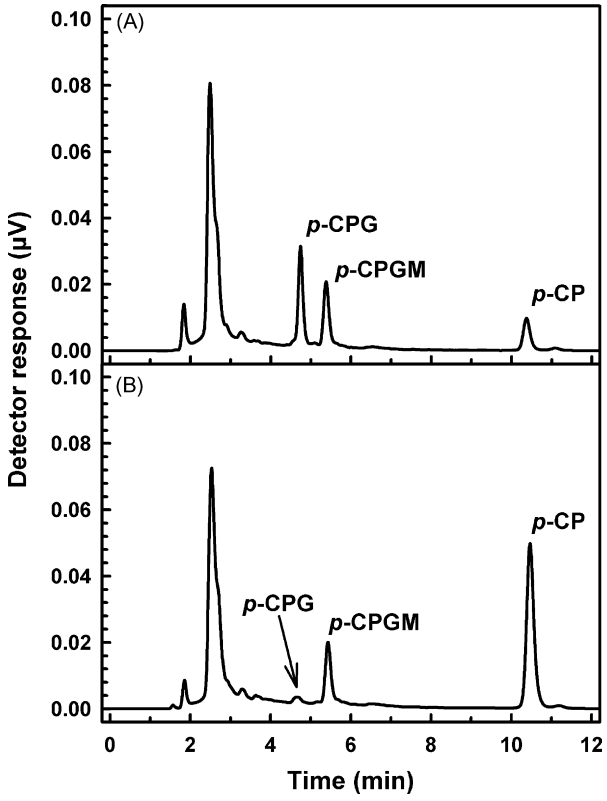

Fig. 6. HPLC analysis of the two metabolites of *p*-CP before (A) and after (B) treatment with β-glucosidase.

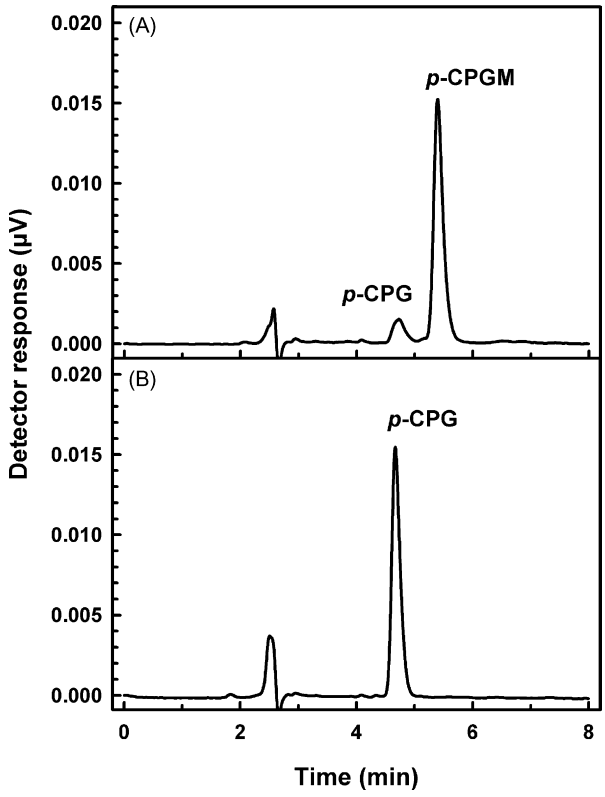

Fig. 7. HPLC analysis of the two metabolites of *p*-CP before (A) and after (B) alkaline hydrolysis.

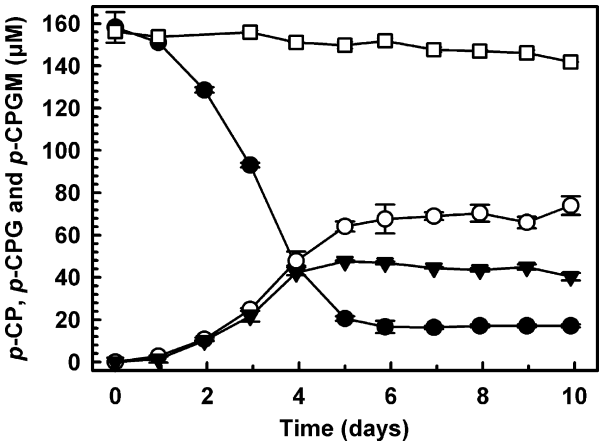

Fig. 8. *p*-CP removal (●) with *p*-CPG (○) and *p*-CPGM (▼) formation during growth of *T. marina* in the presence of 155 μM *p*-CP. Abiotic loss of *p*-CP in growth medium without microalgal cells is also presented (□). The light intensity was 60 μE m^{-2} s^{-1}, the culture temperature was 20 °C, and the aeration rate was 0.6 vvm (error bars indicate standard deviations; $n = 3$).

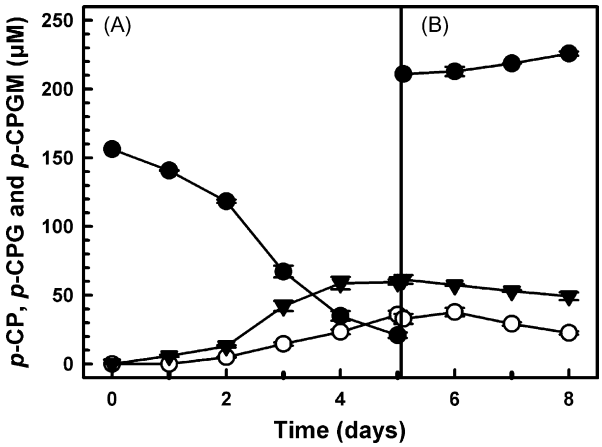

Fig. 10. *p*-CP metabolism (●) with *p*-CPG (○) and *p*-CPGM (▼) formation during growth of *T. marina* in the presence of 155 μM *p*-CP (A). On the fifth day of culture microalgal cells were removed and the supernatant containing possible bacterial contaminants was returned to new columns and spiked with 210 μM *p*-CP. The fate of *p*-CP, *p*-CPG and *p*-CPGM was monitored for three more days (B). The light intensity was 60 μE m^{-2} s^{-1}, the culture temperature was 20 °C, and the aeration rate was 0.6 vvm (error bars indicate standard deviations; $n = 3$).

3.4. Degradation of p-CP by bacteria

Although all the experiments performed in this study started with axenic cultures, it was possible that bacteria might occur in the cultures where the periodic sampling events occurred. In order to determine possible contribution of bacteria to *p*-CP removal from *T. marina* cultures, we conducted an experimental assay as follows. *T. marina* cells were first maintained in the growth medium spiked with 155 μM *p*-CP for five days to allow almost complete deprivation of *p*-CP from the medium (Fig. 10A). The culture was then harvested and centrifuged at 3000 × *g* for 5 min to remove *T. marina* cells. The resulting supernatant which contained bacteria (as confirmed by light microscopy) but not *T. marina* cells was again spiked with 210 μM *p*-CP. The supernatant containing *p*-CP was re-inoculated to the column and maintained under the same culture conditions for three days when the concentrations of *p*-CP, *p*-CPG and *p*-CPGM were monitored daily (Fig. 10B). It revealed that the *p*-CP concentration did not decrease but increased slightly (14.9 ± 1.6 μM) over the three-day period. The slight increase in *p*-CP concentration was observed to be associated with the decrease in both *p*-CPG (10.3 ± 3.7 μM) and *p*-CPGM (17.1 ± 3.2 μM) during the three-day incubation suggesting that the bacteria partially degraded *p*-CPG and *p*-CPGM to release glucose which might be in turn utilized by the bacteria as a carbon/energy source. Therefore, it was concluded that the removal of *p*-CP from *T. marina* cultures was due to the uptake and metabolism of *p*-CP by *T. marina* under our experimental conditions.

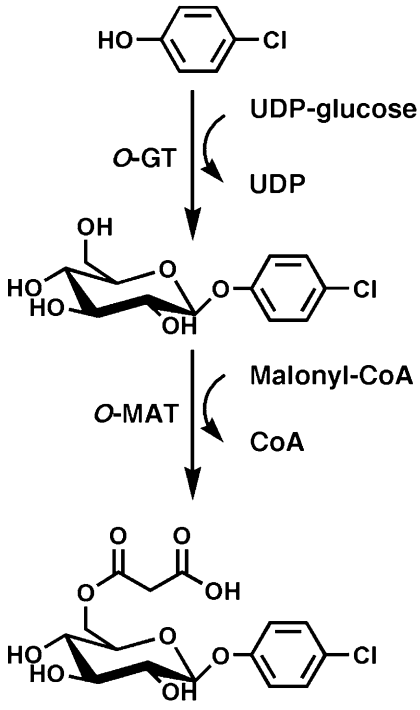

Fig. 9. *p*-CP metabolism as observed in *T. marina*. *O*-GT: *O*-glucosyltransferase, *O*-MAT: *O*-malonyltransferase.

3.5. Photosynthesis-dependence of p-CP metabolism

Uptake and metabolism of *p*-CP by *T. marina* as a function of photo-regime were investigated. *T. marina* cultures were first maintained under continuous illumination until the cell density reached ca. 0.7 g dry wt L^{-1}. The cultures were then spiked with 310 μM of *p*-CP and subjected either to continuous light or a 12 h/12 h light/dark cycle, or to the dark. The effect of light availability and different photo-regimes on *p*-CP removal and concomitant formation of *p*-CPG and *p*-CPGM is shown in Fig. 11. It revealed that the rates of *p*-CP removal and *p*-CPG and *p*-CPGM formation varied greatly among the three treatments. The *p*-CP removal rate was calculated to be −1.72 ± 0.06 μM *p*-CP h^{-1} under continuous illumination, whereas −0.25 ± 0.03 μM *p*-CP h^{-1} was obtained in cultures maintained in the dark. When cultures were main-

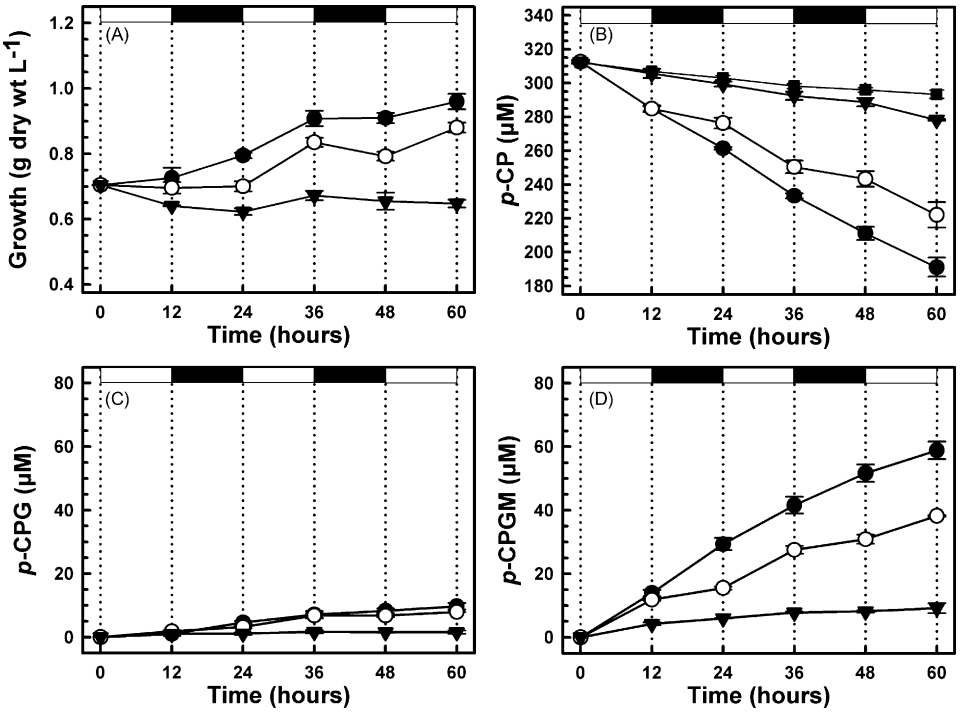

Fig. 11. Photosynthesis dependence of *p*-CP metabolism. *T. marina* cells, previously grown under continuous illumination up to 0.7 g DCW L^{-1}, were spiked with 310 μM of *p*-CP. Growth of microalgae (A) with *p*-CP metabolism (B) and *p*-CPG (C) and *p*-CPGM formation (D) were monitored under continuous light (●), continuous dark (▼) and light: dark 12:12 photoregime (○). Abiotic loss of *p*-CP is also shown (■). Bars at the top indicate the period of illumination (white bar) and darkness (black bar) in LD cycle. The light intensity was 60 μE m^{-2} s^{-1}, the culture temperature was 20 °C, and the aeration rate was 0.6 vvm (error bars indicate standard deviations; $n = 3$).

tained in the 12 h/12 h light/dark photo-regime, a removal rate of -1.8 ± 0.2 μM *p*-CP h^{-1} was measured at the light regime while a rate of -0.4 ± 0.1 μM *p*-CP h^{-1} was detected in the dark period. It appeared that abiotic loss of *p*-CP h^{-1} under light or dark conditions was not significantly different, suggesting that the slight loss of *p*-CP during the cultivation was due to *p*-CP volatilization rather than photo-oxidation.

4. Discussion

Chlorophenolic compounds have been found to be toxic to many marine organisms, including phytoplankton (Borthwich and Schimmel, 1978; Hattula et al., 1981; Kuiper and Hanstveit, 1984; Grimwood and Mascarenhas, 1997). The inhibition constant EC_{50} of 272 ± 17 μM for *p*-CP in *T. marina* under our experimental conditions falls within the range of *p*-CP EC_{50} values reported previously with other freshwater and marine algae, i.e. 297 and 227 μM for *Selenastrum capricornutum* and *Chlorella vulgaris*, respectively (Shigeoka et al., 1988), 65 μM for *Scenedesmus subspicatus* (Kühn and Pattard, 1990), 391 μM for *Chlorella pyrenoidosa* (Huang and Gloyna, 1968), 75 μM for *Phaeodactylum tricornutum* (Kuiper, 1982) and 26 μM for *Skeletonema costatum* (USEPA, 1980).

In higher plants, metabolism of xenobiotics is known to proceed in three phases: Phase I (transformation), phase II (conjugation), and phase III (compartmentation) (Sandermann, 1992). Phase I reactions involve oxidations catalysed by the cytochrome P-450 system. In phase II, in general, the xenobiotic or a phase I-activated metabolite is deactivated by covalent linkage to an endogenous hydrophilic molecule, such as glucose, malonate or glutathione, to form a water-soluble conjugate. In general, phase II products are either nontoxic or less toxic than the parent compound (Coleman et al., 1997). Finally, phase III metabolism either converts phase II metabolites to insoluble residues or conjugates them to an additional molecule (Hall et al., 2001).

Metabolism of xenobiotics in microalgae is less understood. A freshwater green alga *S. capricornutum* degraded polyaromatic hydrocarbon benzo(*a*)pyrene via a dioxygenase pathway and the metabolites were then conjugated to sulfate and glucose (Warshawsky et al., 1990). In contrast, the diatom *Skeletonema costatum* was able to detoxify 2,4-dichlorophenol by conjugation to glutathione (γ-glutamylcysteinylglycine) catalyzed by glutathione S-transferase (Yang et al., 2002). To the best of our knowledge no information about glucosidation of xenobiotics is available in marine microalgae.

In this study, we demonstrate that *O*-glucosylation with subsequent 6-*O*-malonylation of the glucoconjugate was the route of *p*-CP metabolism in *T. marina*. The combination of glucosyl transfer and malonyl transfer has previously been reported in plants in the case of a number of xenobiotics (Cole and Edwards, 2000). Here, we present the first evidence that this metabolic pathway is also present in microalgae. The finding of the occurrence of phase II metabolism of chlorophenols in a marine prasinophyte extends our knowledge of chlorophenol metabolism in the marine environment.

Since both *p*-CPG and *p*-CPGM are more hydrophilic than the parent compound (*p*-CP), they may have a decreased ability

to partition in cell membranes and consequently be less toxic (compared to *p*-CP). Thus, *T. marina* may use glucosidation and malonylation reactions as a survival strategy against the toxic effects of *p*-CP. Indeed, reduction in toxicity of xenobiotics increasing solubility of their derivatives through phase II reactions has been found to occur in some plant species (Coleman et al., 1997). The secretion of *p*-CPG and *p*-CPGM from *T. marina* cells into the environment may represent the ultimate strategy by which *T. marina* gets rid of *p*-CP and perhaps other toxic chlorophenol compounds. The fate of those two metabolites in the marine environment remains an open issue at this point. DCPG and DCPGM could be hydrolyzed and possibly release free *p*-CP, as it is demonstrated in the present study. DCPG and DCPGM could also be directly used as carbon and energy source by heterotrophic microorganisms.

Tsuji et al. (2003) showed that *Chlorella fusca* var. *vacuolata* removed 2,4-dichlorophenol in the light but not in the dark. On the contrary, *Chlorella* VT-1 metabolizes phenol in the dark but not in the light (Scragg, 2006). However, the metabolic pathway of the toxicants was not elucidated in these studies. Thies et al. (1996) reported that the P450-mediated *N*-demethylation of metflurazon by *Chlorella fusca* and *Chlorella sorokiniana* was light dependent, presumably due to formation of $NADPH + H^+$ by photosynthesis, the essential co-factor of P450 monooxygenases. In the present study, the metabolism of *p*-CP by *T. marina* was found to be mainly a photosynthesis-driven process.

5. Conclusion

In conclusion, the present study demonstrates that *T. marina*, a marine prasinophyte, has the ability to metabolize *p*-chlorophenol. The pathway of *p*-CP metabolism in *T. marina* involves an initial conjugation of *p*-CP to glucose to form *p*-chlorophenyl-β-D-glucopyranoside, followed by acylation of the glucoconjugate to form *p*-chlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside, representing the first evidence that this metabolic pathway occurs in microalgae in the presence of chlorinated phenols.

Acknowledgements

We thank warmly Dr. Daniel Brune (Department of Chemistry and Biochemistry, Arizona State University, Tempe, USA) for his assistance in collecting and interpreting the mass spectra.

This work was a part of the research project “Iraklitos” and was co-funded by the European Social Fund and Greek National Resources. A research assistantship was provided to D. Petroutsos by Arizona State University.

References

Araki, H., Tatarazako, N., Kishi, K., Kuroda, K., 2000. Evaluation of bioaccumulation potential of 3,4,5-trichloroguaiacol in a zooplankton (Daphnia magna) by pyrolysis-GC/MS in the presence of tetramethylammonium hydroxide (TMAH). J. Anal. Appl. Pyrolysis 55, 69–80.

Borthwich, P.W., Schimmel, A.C., 1978. Toxicity of pentachlorophenol and related compounds to early life stages of selected estuarine animals. Environ. Sci. Res. 12, 141–146.

Cole, D.J., Edwards, R., 2000. Secondary metabolism of agrochemicals in plants. In: Roberts, T. (Ed.), Metabolism of Agrochemicals in Plants. John Wiley & Sons Ltd., Chichester, England, pp. 107–154.

Coleman, J.O.D., Blake-Kalff, M.M.A., Davies, T.G.E., 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trends Plant Sci. 2, 144–151.

da Costa, A.C.A., de França, F.P., 1998. The behaviour of the microalgae *Tetraselmis chuii* in cadmium-contaminated solutions. Aquacult. Int. 6, 57–66.

Day, J.A., Saunders, F.M., 2004. Glycosidation of chlorophenols by *Lemna minor*. Environ. Toxicol. Chem. 23, 613–620.

Dimou, A.D., Sakellarides, T.M., Vosniakos, F.K., Giannoulis, N., Leneti, E., Albanis, T., 2006. Determination of phenolic compounds in the marine environment of Thermaikos Gulf, Northern Greece. Int. J. Environ. Anal. Chem. 86, 119–130.

Ding, J., Wang, X., Zhang, T., Li, Q., Luo, M., 2006. Optimization of RP-HPLC analysis of low molecular weight organic acids in soil. J. Liq. Chromatogr. R. T. 29, 99–112.

Fleming, B.I., 1995. Organochlorine in perspective. Tappi J. 78, 93–98.

Grimwood, M., Mascarenhas, R., 1997. Proposed Environmental Quality Standards for 2-, 3- and 4-Chlorophenol and 2,4-Dichlorophenol in Water. Environment Agency Technical Report P46/i688. WRc Report No. EA4215.

Grossert, J.S., Fancy, P.D., White, R.L., 2005. Fragmentation pathways of negative ions produced by electrospray ionization of acyclic dicarboxylic acids and derivatives. Can. J. Chem. 83, 1878–1890.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 26–60.

Hall, J.C., Wickenden, J.S., Kerrm, Y.F.Y., 2001. Biochemical conjugation of pesticides in plants and microorganisms: an overview of similarities and divergences. In: Hall, J.C., Hoagland, R.E., Zablotowicz, R.M. (Eds.), Pesticide Biotransformation in Plants and Microorganisms: Similarities and Divergences, ACS Symposium Series 777. American Chemical Society, Washington, DC, pp. 89–118.

Hattula, M.L., Wasenius, M., Reunanen, H., Arstula, V., 1981. Acute toxicity of some chlorinated phenols, catechols and cresols to trout. Bull. Environ. Contam. Toxicol. 26, 295–298.

Hauch, R.G., Norris, D.R., Pierce, R.H., 1980. Acute and chronic toxicity of sodium pentachlorophenate to the copepod, *Pseudodiaptomus coronatus*. Bull. Environ. Contam. Toxicol. 25, 562–568.

Hu, Q., Westerhoff, P., Vermaas, W., 2000. Removal of nitrate from groundwater by cyanobacteria: quantitative assessment of factors influencing nitrate uptake. Appl. Environ. Microb. 61, 133–139.

Huang, J.-C., Gloyna, E.F., 1968. Effect of organic compounds on photosynthetic oxygenation. I. Chlorophyll destruction and suppression of photosynthetic oxygen production. Water Res. 2, 347–366.

Jennings, J.G., de Nys, R., Charlton, T.S., Duncan, M.W., Steinberg, P.D., 1996. Phenolic compounds in the nearshore waters of Sydney, Australia. Mar. Freshwater Res. 47, 951–959.

Koike, K., Jimbo, M., Sakai, R., Kaeriyama, M., Muramoto, K., Ogata, T., Maruyama, T., Kamiya, H., 2004. Octocoral chemical signaling selects and controls dinoflagellate symbionts. Biol. Bull. 207, 80–86.

Kot-Wasik, A., Dębska, J., Namieśnik, J., 2004. Monitoring of organic pollutants in coastal waters of the Gulf of Gdańsk, Southern Baltic. Mar. Pollut. Bull. 49, 264–276.

Kühn, R., Pattard, M., 1990. Results of the harmful effects of water pollutants to green algae (*Scenedesmus subspicatus*) in the cell multiplication test. Water Res. 24, 31–38.

Kuiper, J., 1982. The Use of Model Ecosystems for the Validation of Screening Tests for Biodegradation and Acute Toxicity. Netherlands Organization for Applied Scientific Research. For: CEC, TNO Report CL 82/01.

Kuiper, J., Hanstveit, A.O., 1984. Fate and effects of 4-chlorophenol in marine plankton communities in experimental enclosures. Ecotox. Environ. Safe. 8, 15–33.

Lamoureux, G.L., Rusness, D.G., 1986. Xenobiotic conjugation in higher plants, In: Paulson, G.D., Caldwell, J., Hutson, D.H., Menn, J.J. (Eds.), Xenobiotic Conjugation Chemistry, ACS Symposium Series 299. American Chemical Society, Washington, DC, pp. 62–105.

Laurent, F., Debrauwer, L., Rathahao, E., Scalla, R., 2000. 2,4- Dichlorophenoxyacetic acid metabolism in trasgenic tolerant cotton (*Gossypium hirsutum*). J. Agric. Food Chem. 48, 5307–5311.

Lee, L.S., Rao, P.S.C., Brusseau, M.L., 1991. Nonequilibrium sorption and transport to neutral and ionized chlorophenols. Environ. Sci. Technol. 25, 722–729.

Lovell, C.R., Eriksen, N.T., Lewitus, A.J., Chen, Y.P., 2002. Resistance of the marine diatom *Thalassiosira* sp. to toxicity of phenolic compounds. Mar. Ecol. Prog. Ser. 229, 11–18.

Mcleese, D.W., Zitko, V., Peterson, M.R., 1979. Structure–lethality relationships for phenols, anilines and other aromatic compounds in shrimps and clams. Chemosphere 8, 53–57.

Motulsky, H.J., Christopoulos, A., 2003. Fitting models to biological data using linear and non linear regression. In: A Practical Guide to Curve Fitting. GraphPad Software Inc., San Diego, CA, www.graphpad.com.

OECD, 1984. Test 201: alga, growth inhibition test. OECD Guidelines Testing Chem. 1, 1–14.

Pascal-Lorbel, S., Rathahao, E., Cravedi, J.-P., Laurent, F., 2003. Uptake and metabolic fate of [^{14}C]-2,4-dichlorophenol and [^{14}C]-2,4-dichloroaniline in wheat (*Triticum aestivum*) and soybean (*Glycine max*). J. Agric. Food Chem. 51, 4712–4718.

Petroutsos, D., Katapodis, P., Christakopoulos, P., Kekos, D., 2007. Removal of *p*-chlorophenol by the marine microalga *Tetraselmis marina*. J. Appl. Phycol. 19, 485– 490.

Pflugmacher, S., Sandermann Jr., H., 1998. Taxonomic distribution of glucosyltransferases acting on xenobiotics. Phytochemistry 49, 507–511.

Pridham, J.B., 1964. The phenol glucosylation reaction in the plant kingdom. Phytochemistry 3, 493–497.

Renberg, L., Svanberg, O., Bengtsson, B.-E., Sundström, G., 1980. Chlorinated guaiacols and catechols bioaccumulation potential in bleaks (*Alburnus alburnus*, Pisces) and reproductive and toxic effects on the harpacticoid *Nitocra spinipes* (Crustacea). Chemosphere 9, 143–150.

Sandermann, H., 1992. Plant metabolism of xenobiotics. TIBS 17, 82–84.

Schmitt, R., Kaul, J., v.d. Trenck, T., Schaller, E., Sandermann, H., 1985. β-D-glucosyl and *O*-malonyl-β-D-glucosyl conjugates of pentachlorophenol in soybean and wheat: identification and enzymatic synthesis. Pestic. Biochem. Physiol. 24, 77–85.

Scragg, A.H., 2006. The effect of phenol on the growth of *Chlorella vulgaris* and *Chlorella* VT-1. Enzyme Microb. Tech. 39, 796–799.

Sharma, H.A., Barber, J.T., Ensley, H.E., Polito, M.A., 1997. A comparison of the toxicity and metabolism of phenol and chlorinated phenols by *Lemna gibba*, with special reference to 2,4,5-trichlorophenol. Environ. Toxicol. Chem. 16, 346–350.

Shigeoka, T., Sato, Y., Takeda, Y., Yoshida, K., Yamauchi, F., 1988. Acute toxicity of chlorophenols to green algae, *Selenastrum capricornutum* and *Chlorella vulgaris*, and quantitative structure–activity relationships. Environ. Toxicol. Chem. 7, 847–854.

Thies, F., Backhaus, T., Bossmann, B., Grimme, L.H., 1996. Xenobiotic biotransformation in unicellular green algae – involvement of cytochrome P450 in the activation and selectivity of the pyridazinone pro-herbicide metflurazon. Plant Physiol. 112, 361–370.

Tsuji, N., Hirooka, T., Nagase, H., Hirata, K., Miyamoto, K., 2003. Photosynthesis-dependent removal of 2,4-dichlorophenol by *Chlorella fusca* var. *vacuolata*. Biotechnol. Lett. 25, 241–244.

USEPA (U.S. Environmental Protection Agency), 1980. Ambient water quality criteria for chlorinated phenols. 440/5-80-032. In: Office of Water Regulations and Standards Criteria and Standards Division. U.S. Environmental Protection Agency, Washington, DC.

Van Dyk, J.J., van der Meer, C., Wignans, M., 1977. The toxicity of sodium pentachlorophenate for three species of decapod crustaceans and their larvae. Bull. Environ. Contam. Toxicol. 17, 622–630.

Warshawsky, D., Keenan, T.H., Reilman, R., Cody, T.E., Radike, M.J., 1990. Conjugation of benzo[*a*]pyrene metabolites by freshwater green alga *Selenastrum capricornutum*. Chem. Biol. Interact. 74, 93–105.

Webb, P.W., Brett, W.R., 1973. Effects of sublethal concentration of sodium pentachlorophenate on growth rate, food conversion efficiency and swimming performance in underyearling Sockeye Salmon (*Oncorhynchus nerka*). J. Fish. Res. Board Canada 30, 499–507.

WHO (World Health Organisation), 1989. Chlorophenols other than pentachlorophenol. Environ Health Criteria 93, Geneva, Switzerland.

Yang, S.Y., Wu, R.S.S., Kong, R.Y.C., 2002. Biodegradation and enzymatic responses in the marine diatom *Skeletonema costatum* upon exposure to 2,4-dichlorophenol. Aquat. Toxicol. 59, 191–200.

Zhu, C.J., Lee, Y.K., 1997. Determination of biomass dry weight of marine microalgae. J. Appl. Phycol. 9, 189–194.