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Detoxification of 2,4-dichlorophenol by the marine microalga *Tetraselmis marina*

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Abstract

Xenobiotic chlorinated phenols have been found in fresh and marine waters and are toxic to many aquatic organisms. Metabolism of 2,4-dichlorophenol (2,4-DCP) in the marine microalga *Tetraselmis marina* was studied. The microalga removed more than 1 mM of 2,4-DCP in a 2 l photobioreactor over a 6 day period. Two metabolites, more polar than 2,4-DCP, were detected in the growth medium by reverse phase HPLC and their concentrations increased at the expense of 2,4-DCP. The metabolites were isolated by a C8 HPLC column and identified as 2,4-dichlorophenyl-β-D-glucopyranoside (DCPG) and 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (DCPGM) by electrospray ionization-mass spectrometric analysis in a negative ion mode. The molecular structures of 2,4-DCPG and 2,4-CPGM were further confirmed by enzymatic and alkaline hydrolyses. Thus, it was concluded that the major pathway of 2,4-DCP metabolism in *T. marina* involves an initial conjugation of 2,4-DCP to glucose to form 2,4-dichlorophenyl-β-D-glucopyranoside, followed by acylation of the glucoconjugate to form 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside. The microalga ability to detoxify dichlorophenol congeners other than 2,4-DCP was also investigated. This work provides the first evidence that microalgae can use a combined glucosyl and malonyl transfer to detoxify xenobiotics such as dichlorophenols.

Keywords: *Tetraselmis marina*; Marine microalgae; Xenobiotic metabolism; Detoxification; Chlorophenols

1. Introduction

Chlorophenols are an important class of xenobiotics that have been extensively used in the production of pesticides, herbicides, and wood preservatives. They are listed as priority environmental pollutants by the US EPA because of their high toxicity, carcinogenicity, and persistence (ATSDR, 2005). 2,4-Dichlorophenol (2,4-DCP) is mainly used in the production of the herbicide 2,4-D. Although its use has been strictly restrained there is still a great amount of 2,4-DCP containing wastewater being drained into the marine environment every year (Pereira et al., 1988). The presence of 2,4-DCP in marine environments has been demonstrated (Dimou et al., 2006; Kot-Wasik et al., 2004).

Chlorophenols exert a toxic effect on microorganisms by disrupting energy transduction, either by uncoupling oxidative and photosynthetic phosphorylation or by inhibiting electron transport (Escher et al., 1996; Tissut et al., 1987). Their toxicity increases with the degree of chlorination of the phenol ring. Thus, the pollution of coastal sea waters by chlorophenols could modify the biota of this environment (Fleming, 1995). In a model seawater ecosystem with North Sea coastal plankton communities, 1.0 mg l^{-1} of 2,4-dichlorophenol or 4-chlorophenol significantly inhibited growth of phytoplankton and zooplankton and also caused changes in species composition of the

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phytoplankton community (Kuiper and Hanstveit, 1984). Depending on the species and chlorophenol tested, LC_{50} values that have been reported range between 0.002 to 9.8 mg l^{-1} for marine molluscs, marine crustaceans and marine fish (USEPA, 1980).

Higher plant species have been shown to metabolize a broad range of xenobiotics. Metabolism and detoxification of xenobiotics in plants can generally be divided into three phases: In Phase I (transformation) the xenobiotic may be oxidized, reduced or hydrolysed to introduce or reveal a functional group. In Phase II (conjugation) the xenobiotic or the phase I-activated metabolite is conjugated to glucose, malonate or glutathione by the respective transferases to form a water-soluble conjugate. Finally, phase III metabolism (compartmentation) either converts phase II metabolites to insoluble residues or conjugates them to an additional molecule (Sandermann, 1992; Coleman et al., 1997; Hall et al., 2001).

In the case of phenolic xenobiotics, their metabolism in higher plants most commonly proceeds with the formation of β-*O*-glucosides by the action of uridine diphosphate (UDP)-glucosyltransferases. The glucosylated phenols frequently undergo further metabolism forming 6-*O*-malonate hemi-ester glucose conjugates by the action of malonyl CoA transferase on glucose conjugates in the presence of malonyl CoA (Lamoureux and Rusness, 1986). There is evidence that malonate addition is an important determinant for vacuolar targeting (Edwards and Gatehouse, 1999).

Glucosidation of chlorophenols and/or subsequent malonylation have been mostly described in terrestrial angiosperms – soybean, wheat and cotton being some examples (Schmitt et al., 1985; Pascal-Lorbel et al., 2003; Laurent et al., 2000) but 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 (freshwater and marine) no glucosylation of the phenolic compounds quinol and resorcinol was observed. Later Pflugmacher and Sandermann (1998) showed that several marine macroalgae had *O*-glucosyltransferase activities on chlorophenols. Given the widespread distribution and ecological importance of marine microalgae as primer producers and basis of the food chain it is important to determine their metabolic response to chlorophenols.

Data concerning chlorophenol metabolism by marine microalgae is limited to diatoms. *Thalassiosira* sp. degraded phenol exhibiting both protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase (*ortho*- and *meta*-cleavage) 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). Yang et al. (2002) suggested that the principal mechanism involved in the degradation of 2,4-dichlorophenol in *Skeletonema costatum* was glutathione conjugation since addition of glutathione to the diatom culture enhanced biodegradation with no significant increase in biomass.

Tetraselmis marina is a costal green microalga. Its ability to grow on and remove monosubstituted chlorophenols, with a higher efficiency to the *para*-substituted one (*p*-chlorophenol), from a marine medium has been previously shown. In closed static flasks and under continuous illumination with 1 g l^{-1} $NaHCO_3$ initial concentration *T. marina* removed 65% of 20 mg l^{-1} in a 10 day cultivation period (Petroutsos et al., 2007).

The aim of the present work was to study the metabolism and detoxification of 2,4-dichlorophenol by *T. marina* in a 2 l photobioreactor. The microalga ability to metabolise other dichlorophenol isomers was also investigated.

2. Results

2.1. Growth of T. marina and 2,4-DCP metabolism in a photobioreactor

The results presented in Fig. 1 show that in the absence of 2,4-DCP the experimental system chosen could sustain microalgal growth up to 3.4 g dry wt l^{-1} in a 10 day time interval. The specific growth rate (μ) was calculated to be 0.26 day^{-1}. In order to study the 2,4-DCP metabolism by *T. marina*, the microalga was grown in the photobioreactor under the same experimental conditions. When biomass reached 0.42 g dry wt l^{-1} (after 75 h), 2,4-DCP was added in a fed batch mode. The concentration of the first two 2,4-DCP doses was low (ca. 110 μM) in order to prevent any possible inhibition of *T. marina* growth while the concentration of the next 2,4-DCP doses increased proportionally with the increase of biomass. The microalga showed a high efficiency in removing the dichlorophenol (320 μM DCP in less than 21 h). The addition of 2,4-dichlorophenol did not result in decrease of the specific growth rate compared with the control culture ($\mu = 0.30$ day^{-1}), the final biomass production however was more than 30% lower.

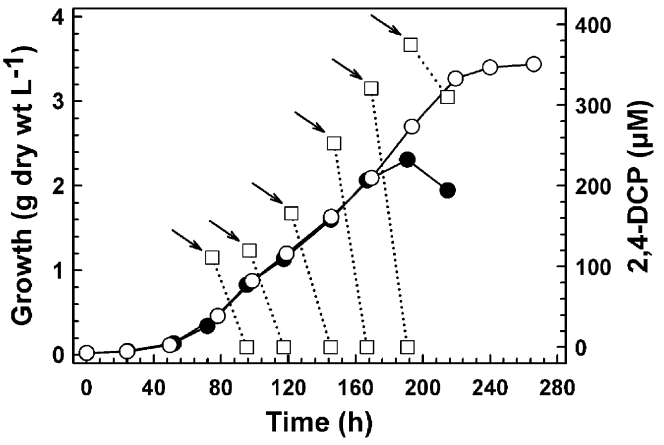

Fig. 1. Growth of *T. marina* in the photobioreactor in the presence (●) and absence (○) of 2,4-DCP. Removal of 2,4-DCP (□) is also presented. Arrows indicate 2,4-DCP addition. Each point represents the average of three measurements. The relative standard deviation of replicate measurements was lower than 1.5% in all cases.

During growth of *T. marina* in the presence of 2,4-DCP two metabolites, named P1 and P2, were formed and accumulated in the growth medium at the expense of 2,4-DCP (Figs. 2a and b). The two metabolites were more polar than the parent compound and their formation was concomitant with the disappearance of dichlorophenol.

P1 and P2 were isolated as described earlier and analyzed using negative ESI-MS. Chlorine signatures (multiple peaks separated by two mass units) were present in all prominent peaks indicating that P1 and P2 metabolites structure contained the parent compound 2,4-dichlorophenol (*m/z* 162). For each peak the degree of chlorination was determined by comparison of percent relative abundance of $m+2$ intensities to theoretical values based on the natural abundance of $^{35}Cl/^{37}Cl$. The ESI-MS spectral data of the two metabolites is presented in Table 1.

For P1, the *m/z* 647 peak with four chlorine atoms was considered to be a dimer of *m/z* 324 (two chlorine atoms) while for *m/z* peak 383 (two chlorine atoms) an acetate ion adduct was assigned. A nominal molecular weight of 324 was assigned to this metabolite. For P2 the *m/z* 819 with 4 chlorine atoms was the dimer of *m/z* 410. The observed *m/z* 44 loss, *m/z* 409–365, 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 410.

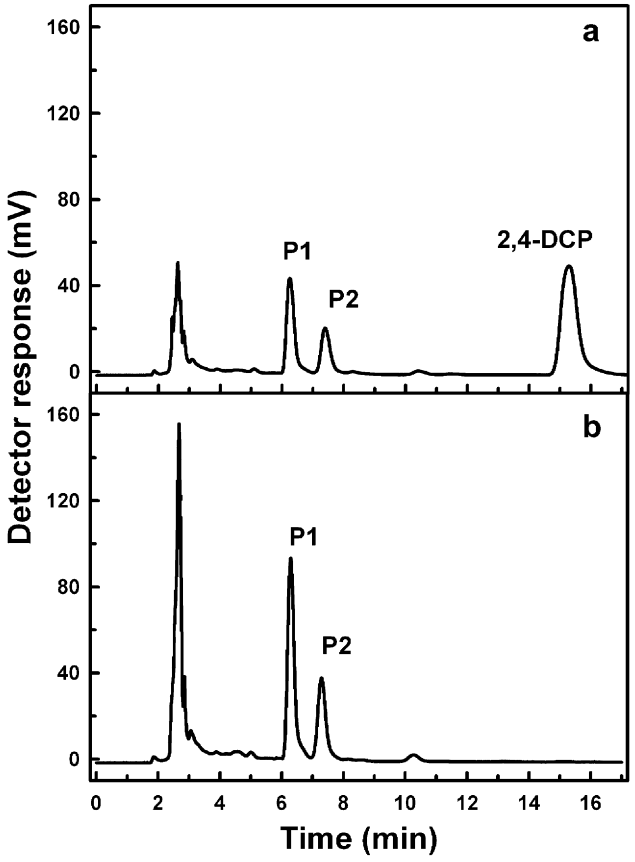

Fig. 2. HPLC profile of 2,4-DCP and its metabolites P1 and P2 obtained from the centrifugate of the culture medium at $t = 135$ h (a) and t = 163 h (b).

Table 1
Mass spectral data of 2,4-dichlorophenol metabolites P1 and P2, obtained by negative ESI-MS

P1		P2	
m/z	Assignment	*m/z*	Assignment
161	$[P1-H-162]^-$	161	$[P2-H-162]^-$
323	$[P1-H]^-$	365	$[P2-H-CO_2]^-$
359	$[P1+Cl]^-$	819	$[P2+P2-H]^-$
383	$[P1+CH_3CO]^-$		
647	$[P1+P1-H]^-$		

The ESI-MS spectral data of P1 and P2 presented here are in full agreement with the ESI-MS spectral data of laboratory prepared 2,4-dichlorophenyl-β-D-glucopyranoside and 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside, respectively (Day and Saunders, 2004). These compounds were formed during metabolism of 2,4-dichlorophenol by the freshwater macrophyte *L. minor*. Thus, P1 was identified as 2,4-dichlorophenyl-β-D-glucopyranoside (DCPG) and P2 was identified as 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (DCPGM).

DCPG and DCPGM structures were confirmed by enzymatic and alkaline hydrolysis. Treatment with β-glucosidase released free 2,4-DCP from DCPG while DCPGM was completely resistant. The release of free glucose in the reaction mixture after the enzymatic hydrolysis was confirmed by high-performance anion exchange chromatography (HPAEC). This is a clear confirmation that DCPG is 2,4-DCP-β-D-glucopyranoside, since β-glucosidase specifically hydrolyses β-D-glucosidic bonds. Alkaline hydrolysis completely cleaved the esteric bond of the malonylated glucoconjugate (DCPGM) and yielded DCPG. The release of malonic acid was also confirmed by HPLC-UV. The time course of DCPG and DCPGM formation at the expense of 2,4-DCP is shown in Fig. 3. DCPG and DCPGM were quantified on the basis of 2,4-DCP release after enzymatic and alkaline hydrolysis.

In conclusion, the major pathway of 2,4-dichlorophenol metabolism and detoxification in *T. marina* involves an initial enzymatic synthesis of a dichlorophenyl-β-D-glucopyranoside conjugate (DCPG) followed by an acylation to form the dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (DCPGM) (Fig. 4).

2.2. Detoxification of other dichlorophenols by T. marina

Microalgal cells, previously grown in the photobioreactor until they reached a density of 3 g dry wt l^{-1} were used to study the detoxification ability of *T. marina* against dichlorophenols besides 2,4-DCP. All six existing dichlorophenol congeners were tested. Four of them (2,3-, 2,4-, 2,5- and 3,4-DCP) were metabolized by more than 90% each

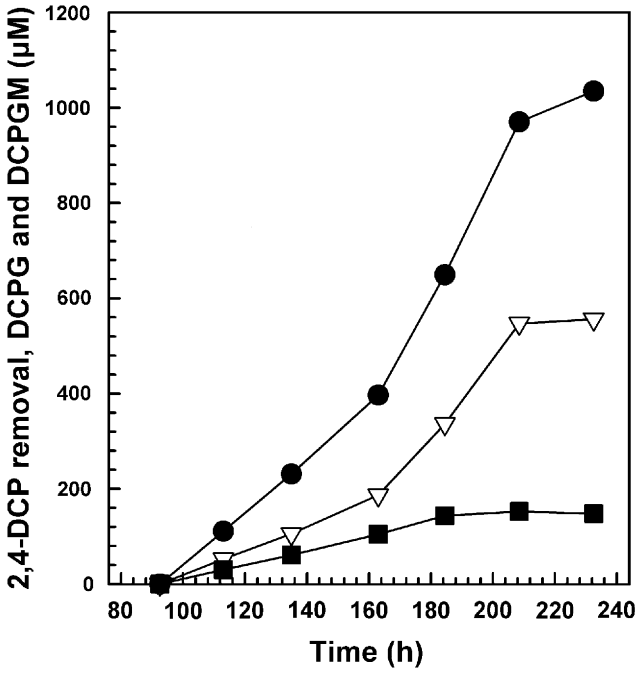

Fig. 3. 2,4-DCP removed (●) in the photobioreactor with DCPG (▽) and DCPGM (■) formation. Each point represents the average of three HPLC measurements. The relative standard deviation of replicate measurements was lower than 1.7% in all cases.

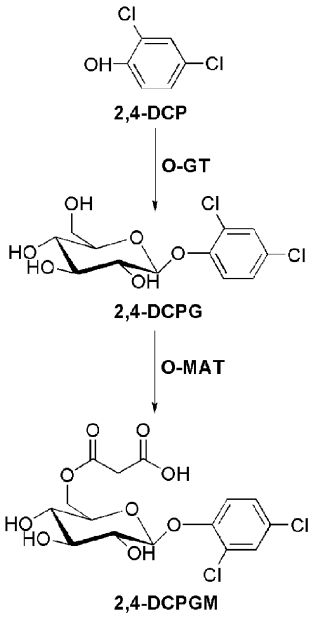

Fig. 4. 2,4-DCP metabolism in *Tetraselmis marina*; O-GT: *O*-glucosyltransferase, O-MAT: *O*-malonyltransferase, 2,4-DCP: 2,4-dichlorophenol, 2,4-DCPG: 2,4-dichlorophenyl-β-D-glucopyranoside, 2,4-DCPGM: 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside. Structures of 2,4-DCPG and 2,4-DCPGM were confirmed by enzymatic (β-glucosidase) and alkaline hydrolysis.

forming one metabolite. All metabolites were isolated and analyzed with ESI-MS. Their MS spectral data were identical with the data obtained from the analysis of 2,4-dichlorophenyl-β-D-glucopyranoside conjugate (DCPG)

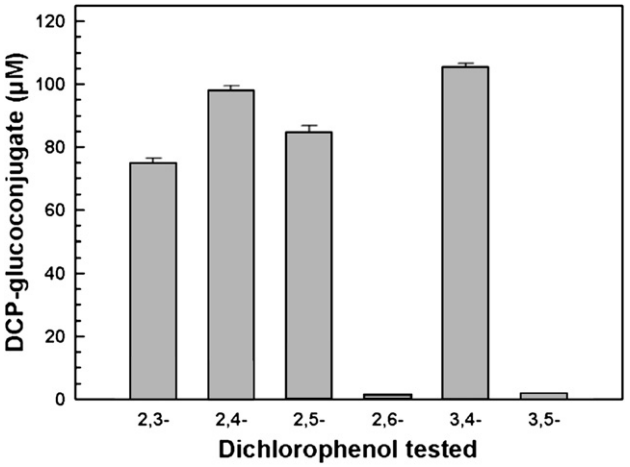

Fig. 5. Dichlorophenols glucosidation by *T. marina*. Microalgal biomass (3 g l^{-1}dry weight) transferred to 10 ml clear serum bottles, received 120 μM of each dichlorophenols and was illuminated for 24 h at 100 μE m^{-2} s^{-1}at 18 °C. (Error bars indicate standard deviations; $n = 2$).

described earlier. The glucosidation of each of the dichlorophenols tested is presented in Fig. 5.

3. Discussion

Our data demonstrate that 2,4-dichlorophenol metabolism in *T. marina* proceeds with subsequent formation of 2,4-dichlorophenyl-β-D-glucopyranoside and 2,4-dichlorophenyl-β-D-(6-*O*-malonyl)-glucopyranoside (phase II metabolites). Xenobiotics conjugation to glucosyl and malonyl-glucosyl moieties appears to occur frequently in plants (Cole and Edwards, 2000); to the best of our knowledge however this metabolic sequence has not been reported earlier in microalgae. Moreover, no other marine microalga has been reported to glucosidate xenobiotics.

Different metabolic responses of microalgae to the presence of xenobiotics have been reported. The freshwater *Selenastrum capricornutum* degraded the polyaromatic hydrocarbon benzo(*a*)pyrene via a dioxygenase pathway and then conjugated the metabolites to sulfate and glucose (Warshawsky et al., 1990). Dioxygenases activities also accounted for the elimination of phenolics in *Ochromonas danica* and *Thalassiosira* sp. (Semple and Cain, 1996; Lovell et al., 2002) while *Skeletonema costatum*, a marine diatom, conjugated 2,4-dichlorophenol to glutathione (Yang et al., 2002). Involvement of P450 monooxygenases in the biotransformation of herbicides in the green alga *Chlorella fusca* and *Chlorella sorokiniana* were reported by Thies et al. (1996). Glucosidation of 2,4-DCP and subsequent malonylation of 2,4-DCP-glucoconjugate by *T. marina* presented here, provides additional evidence on the metabolic versatility microalgae utilize to detoxify xenobiotics.

Our data show that ca. 68% of 2,4-DCP removed resulted in the formation of the two metabolites 2,4-DCPG and 2,4-DCPGM. After disruption of microalgal cells and

HPLC analysis of their intracellular and membrane-bound content no 2,4-DCP was detected either inside or on the surface of microalgal cells and thus biosorption of 2,4-DCP in *T. marina* cells was excluded. The same analysis showed that the summation of the concentrations of the two metabolites inside the cells or bound at the cells surfaces never exceeded 2.9 μmol g dry wt^{-1} or 2% of the concentration of DCPG and DCPGM accumulated in the growth medium. So, the rest 30% of 2,4-DCP removed may be attributed to the formation of other undetected metabolites or to abiotic losses (photooxidation and/or evaporation).

The fact that *T. marina* uses glucosidation and malonylation reactions to convert hydrophobic 2,4-DCP to hydrophilic 2,4-DCPG and 2,4-DCPGM may be to decrease the ability of resulting metabolites to partition in cell membranes and consequently become less toxic to the alga. Indeed, reduction in toxicity of xenobiotics by increasing solubility of their derivatives through phase II reactions has been found to occur in some plant species (Coleman et al., 1997). Detoxification of phenol through conjugation to phenol by the duckweed *Lemna gibba* (a floating aquatic angiosperm) has been reported by Ensley et al. (1996). The conjugate formed, phenyl-β-D-glucopyranoside, has been found to be half as toxic as phenol itself.

Growth of *T. marina* in the presence of 200 μM of 4-CP was inhibited by 50% (Petroutsos et al., 2007) while addition of 200 μM 2,4-DCP at the initial stage of the culture completely inhibited *T. marina* growth. The uninhibited growth of the microalga up to 167 h (Fig. 1) in the presence of 290 μM of 2,4-DCPG and 2,4-DCPGM that were accumulated in the culture medium at the said time (Fig. 3, $t = 167$ h) is a strong indication that through glucosidation and malonylation *T. marina* renders 2,4-DCP less toxic.

However, although 2,4-DCPG and 2,4-DCPGM are less toxic than 2,4-DCP, they will still retain some toxicity. The accumulation of 2,4-DCPG and 2,4-DCPGM in the growth medium together with the presence of unmetabolized amounts of 2,4-DCP in the photobioreactor most likely accounted for the cell lysis observed at 200 h of the culture.

The contamination tests that were routinely conducted in the present study confirmed in all cases the axenicity of microalgal cultures. However, even if bacterial contamination had occurred that would not necessarily mean that bacteria would contribute to chlorophenol detoxification. Xenobiotic glucosidation reactions are dominant in plants where glucose is abundant. It would be metabolically expensive for bacteria to conjugate xenobiotics to glucose for the purpose of detoxification since they are heterotrophs (Hall et al., 2001).

It is important to note, that glucosyl conjugates may be cleaved by glucosidases, and in the case of malonyl-glucosides by esterases. The action of these enzymes will yield the respective aglyca, regenerating the original xenobiotic substrate. This reversibility represents a great disadvantage of glucosylation for its practical consideration in phytoremediation, because previously detoxified compounds may regain their toxicity under certain conditions (Schröder, 2006). Ensley et al. (1996) demonstrated that when 2,4-dichlorophenol-β-D-glucopyranoside contained in duckweed was fed to red swamp crayfish it was rapidly cleaved in the crayfish stomach back to glucose and 2,4-dichlorophenol. *T. marina* cells in order to protect from the toxic action of 2,4-DCP metabolize it to the less toxic DCPG and DCPGM. The fate of those two metabolites however in the marine environment remains an open issue. DCPG and DCPGM could be hydrolyzed releasing glucose and malonic acid that may be used as a source of nutrition by other microbial populations. They could also be directly used as carbon and energy source from heterotrophic microorganisms or finally they could be further detoxified (e.g. dechlorinated) by other microorganisms.

Our data show that the position of the chloro-substituent in the aromatic ring has an important effect on the microalga ability to conjugate glucose to dichlorophenols. No other comparative studies concerning glucosidation of dichlorophenols have been published so far, thus further study will be needed to elucidate any possible correlation between dichlorophenols structure, toxicity and glucosidation susceptibility. Metabolism of 2,4-DCP in the photobioreactor resulted in the formation of two metabolites, DCPG and DCPGM, while in closed bottles 2,4-DCP was transformed to one only metabolite, the glucoconjugate DCPG. This difference should be attributed to the totally different experimental conditions that were used. Most likely, the metabolism of 2,3-, 2,5- and 3,4-DCP in the photobioreactor would result in formation of both the glucosylated (DCPG) and the malonylated (DCPGM) metabolites.

4. Experimental

4.1. General experimental procedures

Dihlorophenols were obtained from Sigma Chemical Ltd. (St. Louis, MO, USA). MeOH HPLC grade was obtained from Labscan Analytical Sciences (Labscan Ltd., Dublin, Ireland). All other chemicals used in this study were from Sigma Chemical Ltd. and were reagent grade or higher purity. A Hitachi U-1100 Spectrophotometer (Hitachi Ltd, Tokyo, Japan) was used for optical density measurements. HPLC analysis was performed using a Jasco PU-987 (Jasco Co., Ltd., Tokyo, Japan) HPLC pump connected with a 4.6x300 mm Spherisorb C_8 column (Waters, Milford, MA, USA) and a Waters 484 UV detector. For high-performance anion exchange chromatography (HPAEC) a 4.0×250 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 were used. Mass spectrometry (MS) was performed using an LCQ Deca equipped with a heated capillary

electrospray interface (Thermo Fisher Scientific, Waltham, MA, USA) ion trap mass spectrometer. The MS electrospray analyses were carried out under automatic gain control conditions, using a needle voltage of 5.0 kV and a heated capillary temperature of 150 °C. The mass spectrometer was interfaced to a computer workstation running BioBrowser software version 2.0 for data acquisition and processing.

4.2. 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, WA, USA). The microalga was grown and maintained in sterile f/2-Si enriched (Guillard, 1975) artificial seawater (in $g\,l^{-1}$: NaCl 26.52, $MgCl_2 \cdot 6H_2O$ 5.21, $MgSO_4 \cdot 7H_2O$ 6.76, $CaCl_2 \cdot 6H_2O$ 2.25, KCl 0.785, $NaHCO_3$ 0.202, NaBr 0.083; Sverdrup et al., 1942) in Erlenmeyer flasks of 100 ml enclosed with cotton plugs, without shaking. Flasks were illuminated at 60 $\mu E\,m^{-2}\,s^{-1}$ by cool-white fluorescent light under a 16:8 h light:dark photoregime at 18 °C. pH was adjusted to 8.0 with 40 mM Tris–HCl prior to autoclaving at 121 °C for 20 min. Stock cultures were transferred to fresh medium every 7 days.

4.3. Experimental system

2,4-Dichlorophenol detoxification was studied in a 2 l thermoregulated double-jacket cylindrical photobioreactor (B. Braun Biotech International, Germany) with working volume of 1.5 l of microalgal culture. The bioreactor was surrounded by 10 dimmable fluorescent lamps (Osram Dulux T/E Plus 32 W/840). Light intensity at the surface of the bioreactor could reach up to 2000 $\mu E\,m^{-2}\,s^{-1}$ as measured with a QMSS Apogee quantum meter (Apogee Instruments Inc., Logan, UT, USA). The bioreactor with 1400 ml of artificial seawater was sterilized at 121 °C for 30 min. After cooling, the artificial seawater was enriched with F/2 medium and was inoculated with 100 ml of 7-day-old static microalgal culture. Light intensity was initially set at 150 $\mu E\,m^{-2}\,s^{-1}$ and was step wise increased with increasing microalgal cell density, finally reaching 1800 $\mu E\,m^{-2}\,s^{-1}$. The fermentation was carried out at 18 °C and agitation speed of 150 rpm and was aerated at 0.05 vvm with 1% (v/v) CO_2 enriched air. Monitoring and additions of phosphate and nitrate in the culture medium ensured that the microalga would not be nutrient limited.

For the study of *T. marina* ability to detoxify dichlorophenols, 5 ml of microalgal biomass, previously grown in the photobioreactor to a cell density of 3 $g\,l^{-1}$dry weight, were transferred to 10 ml clear serum bottles and received 120 μM of each dichlorophenol. The bottles were closed with butyl septa, crimped with aluminum caps, to minimize dichlorophenols evaporation losses and illuminated for 24 h at 100 $\mu E\,m^{-2}\,s^{-1}$at 18 °C. Bottles containing dichlorophenols dissolved in culture medium without algae were used as blanks.

In all experiments dichlorophenols were first properly dissolved in pure methanol and then added to the microalgal culture in order to obtain the final concentrations desired. The volume of methanol added never exceeded 0.2% of the final culture volume.

Purity of the algal suspensions was routinely monitored microscopically and by inoculating 0.5 ml of the algal culture on nutrient agar (Sigma–Aldrich Chemical Co., St. Louis, USA) and incubating for 2 weeks at 25 °C in the dark. Contamination tests were also performed after the experiments.

4.4. Culture growth

Cell density was monitored as the optical density at 750 nm (OD_{750}) of the algal suspension in a 1 cm cuvette. This wavelength was chosen to avoid interference due to absorption of the pigments. The results were quantified in terms of dry cell wt. For dry cell wt determination, an aliquot of 10 ml algal suspension was filtered through 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).

4.5. Analytical methods

For dichlorophenols analysis *T. marina* cells were separated from the culture broth through centrifugation at $12{,}000 \times g$ for 5 min. The supernatant was filtered through a 0.45 μm nitrocellulose filter (GelmanSciences, MI, USA) and the filtrate was further used as the source of dichlorophenols. Analysis was performed by HPLC-UV at 280 nm. 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 ambient temperature. Malonic acid detection was performed at 220 nm with a mobile phase of 10 mM KH_2PO_4–CH_3OH (95:5, pH 2.7), at flow rate of 0.8 $ml\,min^{-1}$ (Ding et al., 2006). Enzymatic hydrolysates were analyzed for neutral monosaccharide content by HPAEC with a mobile phase consisting of isocratic 20 mM NaOH at a flow rate of 1 $ml\,min^{-1}$. For nitrate determination the method described by Diatloff and Rengel (2001) was used. Phosphate was determined using method 4500-P E (ascorbic acid) as described in Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

4.6. Metabolites identification

An aliquot (100 μl) of filtered supernatant of the culture broth was injected to the HPLC system. The metabolites

were separately collected at the UV detector cell outlet, in methanol/water/acetic acid (50/49/1) mixture and were ready for MS analysis. Each analyte was directly infused into the mass spectrometer at a flow rate of 3 μl min^{-1}. The total run time was 5 min and the analysis was performed in a negative ion scan mode and a *m/z*-range set to 80–2000.

4.7. Intracellular content analysis

Samples taken from the photobioreactor were routinely checked for 2,4-DCP, DCPG and DCPGM intracellular content. Samples (15 ml) were centrifuged at $12{,}000 \times g$ for 10 min in order to separate *T. marina* cells from the culture broth. Microalgal cells were washed with artificial seawater twice and they were resuspended afterwards in 15 ml of artificial seawater. The microalgal suspension was disrupted by sonication, and centrifuged at $12{,}000 \times g$ for 10 min to remove cell debris. The efficiency of cell disruption was evaluated in terms of the amount of released protein using the Bradford method (Bradford, 1979). After centrifugation the supernatant was concentrated using a solid phase extraction (SPE) method. The 15 ml aliquot of cell disruption 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. 2,4-DCP metabolites and 2,4-DCP were retained and 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. For cell surface adsorbed 2,4-DCP or membrane-bound DCPG and DCPGM determination, 1 ml of methanol was added to the cell debris (after cell disruption and centrifugation) and the mixture was placed in an Eppendorf Thermomixer (Eppendorf, Westbury, NY, USA) and incubated for 1 h at 30 °C under agitation at 1100 rpm. After centrifugation at $12{,}000 \times g$ for 10 min the supernatant was analyzed by HPLC.

4.8. Enzymatic hydrolysis

Enzymatic hydrolysis was accomplished with β-glucosidase from almonds (3 mg ml^{-1}, specific activity 6 U mg^{-1}, Fluka) for 8 h at 25 °C and pH 5.0 (40 mM phosphate-citrate buffer). 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 earlier.

4.9. 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 directly analyzed by HPLC.

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References

APHA, 1998. 4500-P E., Ascorbic acid method. In: Clesceri, L.S., Greenberg, A.E., Eaton, A.D. (Eds.), Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC, USA, pp. 4-146–4-147.

ATSDR, Agency for Toxic Substances and Disease Registry, 2005. Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of Hazardous Substances. <http://www.atsdr.cdc.gov/cercla/05list.html>.

Bradford, M., 1979. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cole, D.J., Edwards, R., 2000. Secondary metabolism of agrochemicals in plants. In: Roberts, T. (Ed.), Metabolism of Agrochemicals in Plants. John Wiley and 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.

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

Diatloff, E., Rengel, Z., 2001. Compilation of simple spectrophotometric techniques for the determination of elements in nutrient solutions. J. Plant Nutr. 24, 75–86.

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. Envir. 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. Liquid Chromatogr. R. T 29, 99–112.

Edwards, R., Gatehouse, J.A., 1999. Secondary metabolism. In: Lea, P.J., Leegood, R.C. (Eds.), Plant Biochemistry and Molecular Biology. John Wiley, New York, pp. 193–218.

Ensley, H.E., Sharma, H.A., Barber, J.T., Polito, M.A., 1996. Metabolism of chlorinated phenols by *Lemna gibba*, duckweed. In: Kruger, E.L., Anderson, T.A., Coats, J.R. (Eds.), Phytoremediation of Soil and Water Contaminants, ACS Symposium Series, vol. 664. American Chemical Society, Washington, DC, pp. 238–253.

Escher, B.I., Snozzi, M., Schwarzenbach, R.P., 1996. Uptake, speciation and uncoupling activity of substituted phenols in energy transducing membranes. Environ. Sci. Technol. 30, 3071–3079.

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

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, vol. 777. American Chemical Society, Washington, DC, pp. 89–118.

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.

Kuiper, J., Hanstveit, A.O., 1984. Fate and effects of 4-chlorophenol in marine plankton communities in experimental enclosures. Ecotoxicol. Environ. Saf. 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, vol. 299. American Chemical Society, Washington, DC, pp. 62–105.

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

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.

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.

Pereira, W.E., Rostad, C.E., Chlou, C.T., Brinton, T.I., Barber, L.B., 1988. Contamination of estuarine water, biota, and sediment by halogenated organic compounds: a field study. Environ. Sci. Technol. 22, 772–778.

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.

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.

Schröder, P., 2006. Enzymes transferring biomolecules to organic foreign compounds: a role for glucosyltransferase and glutathione *S*-transferase in phytoremediation. In: Mackova, M., Dowling, D., Macek, T. (Eds.), Focus on Biotechnology, Phytoremediation and Rhizoremediation, vol. 9A. Springer, Berlin, Heidelberg, pp. 133–142.

Semple, K.T., Cain, R.B., 1996. Biodegradation of phenols by the alga *Ochromonas danica*. Appl. Environ. Microbiol. 62, 1265–1273.

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

Sverdrup, H.V., Johnson, N.W., Fleming, R.H., 1942. The Oceans. Prentice-Hall Inc., New York, p. 1087.

Thies, F., Backhaus, T., Bossmann, B., Grimme, L.H., 1996. Xenobiotic biotransformation in unicellular green algae. Plant Physiol. 112, 361–370.

Tissut, M., Ravanel, T.R., Benoit-Guyod, J.L., 1987. Effects of chlorophenols on isolated class A chloroplasts and thylakoids: a QSAR study. Environ. Environ. Saf. 13, 32–42.

U.S. Environmental Protection Agency, 1980. Ambient water quality criteria for chlorinated phenols. EPA Report No. 440/5-80-032. Washington, DC

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.

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.