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TOXICITY AND METABOLISM OF 2,4-DICHLOROPHENOL BY THE AQUATIC ANGIOSPERM *LEMNA GIBBA*

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Abstract—The toxicity and metabolism of 2,4-dichlorophenol, with regard to the aquatic macrophyte *Lemna gibba* (duckweed), have been studied Toxicity is described in terms of the effect of 2,4-dichlorophenol (2,4-DCP) on the vegetative reproduction of duckweed over a 10-d growth period, the EC10 and EC50 were 2.5 and 9 2 μM, respectively. Metabolism of 2,4-dichlorophenol was monitored by incubation of the plants with radiolabeled substrate, and periodic sampling and analysis by reversed-phase HPLC of the plant growth medium Depending on the growth conditions, up to 95% of the 2,4-DCP was metabolized over a 6-d growth period. To analyze the metabolites, the plants were grown in the presence of sublethal concentrations of [U-^{14}C]-2,4-DCP. The growth medium was lyophilized and then mixed with the plants, extracted, and analyzed using reversed-phase HPLC, followed by scintillation counting of the fractions The major metabolite was isolated and identified as 2,4-dichlorophenyl-β-D-glucopyranoside by high-field NMR and MS. The structure of the metabolite was confirmed by synthesis and by enzymatic cleavage of the β-glucosidic linkage to afford 2,4-DCP. An important consequence of conjugate formation is the masking of the presence of 2,4-DCP to the usual analytical techniques used for its detection and quantitation. This finding is probably applicable to other contaminants and organisms.

Keywords—Duckweed 2,4-Dichlorophenol Toxicity Metabolism

INTRODUCTION

2,4-Dichlorophenol (2,4-DCP) has extensive industrial and commercial uses and consequently is a contaminant that inevitably invades surface waters. The biological fate of 2,4-DCP is of concern, not only because of its toxicity and direct effects on aquatic ecosystems, but also because of its potential for indirect harm to animal populations as a result of entry into food chains and subsequent bioaccumulation, -modification, and -transport. To assess these issues, the aquatic angiosperm *Lemna gibba* (duckweed) was chosen for study. Members of the Lemnaceae are ubiquitous, ecologically important, easily grown, and readily and reliably manipulated.

Previous studies have evaluated the toxicity and fate of 2,4-DCP, as well as other chlorinated phenols, with regard to animals [1–3] and various plants [4–9]. However, few reports pertain to aquatic plants [5–9], fewer yet to *Lemna* [7–9], and none to *L. gibba* specifically. Unlike *Phanerochaete chrysosporium* [10], higher plants are incapable of mineralizing chlorinated phenols but can modify them in various ways [11–16]. Given the widespread distribution and ecological importance of duckweed, it is important to determine its metabolic response to 2,4-DCP and to isolate and identify the metabolites.

MATERIALS AND METHODS

2,4-DCP and [U-^{14}C]-2,4-DCP (20.2 mCi mmol^{-1}) were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO) chemical companies, respectively, and used without further purification. The labeled 2,4-DCP was diluted 1:10 with absolute ethanol before use. Stocks of *L. gibba* G3 were maintained in sterile culture in 125-ml Erlenmeyer flasks containing 50 ml of the medium described by Cleland and Tanaka [17], plus sucrose and tryptone to reveal microbial contamination. The cultures were grown in chambers under continuous light (2.0×10^{-3} W cm^{-2}; 300–1,100 nm at plant level) at 27.8°C. Experimental cultures, 10 replicates of each, were started using approximately 15 fronds per culture (i.e., 5 three-frond colonies each). The number of fronds in each culture at day 0 was noted. 2,4-DCP was added aseptically from a stock solution to give the following concentrations: 0, 2.5, 6.1, 12.0, 24.0, and 60.0 μM. Experimental cultures were grown under continuous light at a constant 27.8°C. After a 10-d growth period, the fronds were counted in each flask and the percentages of frond increases were calculated according to the formula

$$\frac{\text{No. fronds at day 10} - \text{no. fronds at day 0}}{\text{no. fronds at day 0}} \times 100$$

To investigate the disappearance of 2,4-DCP from the growth medium, the plants were grown, as described above, in the presence of 15 μM 2,4-DCP. At daily intervals, a 100-μl sample of medium was aseptically withdrawn and analyzed with a Hewlett Packard (Avondale, PA) model 5995 GC-MS operated in the selected-ion-monitoring (SIM) mode set to detect m/z 162 and 164. A similar experiment was conducted using radioactive 2,4-DCP (25 μl of the stock [U-^{14}C]-2,4-DCP) as a tracer. At daily intervals, 100 μl medium was re-

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moved and assayed for radioactivity using Ecolume® cocktail (ICN Biomedicals Corp., Irvine, CA) and a Beckman Instruments (Fullerton, CA) LS 6000IC liquid scintillation detector.

To investigate the possible metabolism of 2,4-DCP, experiments were conducted as described above, except that sucrose and tryptone were omitted from the medium, and 2,4-DCP was added at a sublethal concentration (15 μM), along with [^{14}C]-2,4-DCP (0.5 μCi) as a tracer. At 24-h intervals, 1-ml aliquots were withdrawn aseptically from the cultures and analyzed by reversed-phase HPLC (RP-HPLC) using a Waters (Mississauga, Ontario) 600E HPLC system with an Alltech (Australia) analytical column (250 × 4.6 mm i.d.; Adsorbosphere® HS C_{18}, 5 μm). A linear elution gradient was employed using HPLC-grade acetonitrile and deionized distilled water, with a flow rate of 1 ml min^{-1}. The gradient ran over a 30-min interval, starting with a mixture of 10% acetonitrile:90% water, and ending with a mixture of 99% acetonitrile:1% water. One-milliliter fractions were collected and assayed for radioactivity by liquid scintillation counting. The results were plotted as number of counts vs. fraction number, from which the disappearance of 2,4-DCP and the concomitant appearance of the metabolites were evident. The incubation time for maximum production of the major metabolite was determined to be 6 d. With incubation periods of >6 d, an even more polar secondary metabolite became increasingly evident.

The isolation of the major metabolite required large-scale incubations (500 ml medium in 3-L Fernbach flasks) that contained higher concentrations of 2,4-DCP (40 μM) than those used previously and a greater number of fronds to accommodate those higher concentrations. The 2,4-DCP, spiked with radiolabeled substrate, was added to the medium, along with approximately 1,000 fronds. The plants were harvested after 6 d, and the growth medium was lyophilized. The plants were mixed with the lyophilized medium and homogenized in methanol using a Brinkmann Instruments (Westbury, NY) model PT10/35 stainless steel homogenizer. The homogenized mixture was then filtered, and the filtrate was reduced in volume on a Brinkmann model RE 121 rotavapor. The resulting methanol extract was passed through a silica-gel column (60–200 mesh) and eluted first with ethyl acetate and then with methanol. Radioactive assays of the eluate showed that the metabolite was contained in the methanol fraction, which was reduced in volume on the rotavapor and subjected to RP-HPLC using an Alltech semipreparatory column (300 × 10 mm i.d.; Adsorbosphere HS C_{18}, 5 μm). The RP-HPLC parameters were the same as previously described, with the exception that the flow rate was adjusted to 5 ml min^{-1} to compensate for the larger column. One-milliliter fractions were collected and assayed for radioactivity by liquid scintillation counting. The retention time of the radioactive metabolite was determined in this way, and the appropriate fractions were combined and reduced in volume using rotary evaporation; the resulting purified metabolite was analyzed by NMR and GC-MS.

The metabolite was dissolved in acetonitrile-d_3 and analyzed with a Bruker (Karlsruhe, Germany) 200-MHz NMR spectrometer. Silylation of the metabolite using a trimethylsilyl-imidazole-pyridine mixture (Supelco, Bellefonte, PA) was performed before GC-MS analysis using a Kratos Profile® spectrometer (electron impact, 70 eV; 16-m capillary column with DB-1 stationary phase).

The natural metabolite was treated with β-glucosidase (0.1 mg ml^{-1}; specific activity 20 units mg^{-1}; Sigma) at 25°C and pH 5.6 (0.01 M 2-morpholinoethanesulfonate [MES] buffer). The products of the reaction were analyzed by RP-HPLC, as described above, except that a 25-min gradient was used, starting with a mixture of 15% acetonitrile:85% water, and ending with a mixture of 30% acetonitrile:70% water.

The synthesis of the metabolite was accomplished using a modification of the procedure by Sinnott and Souchard [18]: 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.5 g, 6.1 mmol), 2,4-DCP (1.6 g, 9.8 mmol), 1 N NaOH (10 ml, 10 mmol), and acetone (15 ml) were placed in a round-bottom flask and allowed to stir overnight. The acetone was then removed by rotary evaporation, leaving a solid residue. Water (75 ml) was added to the residue, and the mixture was extracted with $CHCl_3$ (3 × 30 ml). The combined extracts were washed with 2 N NaOH (3 × 30 ml) then water (1 × 50 ml), dried with $MgSO_4$, and filtered; the solvent was removed by rotary evaporation. The crude glucoside (1.97 g) was purified by recrystallization from hot ethanol to yield 2,4-dichlorophenyl-β-D-glucopyranoside tetraacetate (1.6 g, 81%) as long white needles.

2,4-Dichlorophenyl-β-D-glucopyranoside was formed by treating the tetraacetate (1.6 g, 49 mmol) with MeONa (29 mg) in methanol (30 ml). The mixture was warmed just to the point where it became a homogeneous solution and was allowed to stir for 1.5 h, at which time 1 N HCl (0.8 ml) was added, followed by evaporation of the solvent. The 2,4-dichlorophenyl-β-D-glucopyranoside was recrystallized from ethanol as white needles (0.20 g, 10%): ^{1}H NMR (200 MHz, CD_3CN) δ 3.4 to 4.6 (multiplet, 10 H), 5.1 (doublet, J = 7.3 Hz, 1 H), 7.4 (multiplet, 3 H).

The synthetic metabolite was silylated and analyzed by GC-MS, using the same conditions as described above for the natural product.

RESULTS AND DISCUSSION

The effect of 2,4-DCP on the vegetative reproduction (percent frond increase) of *L. gibba* is shown in Figure 1. Concentrations >40 μM were lethal. EC10 (2.5 μM) and EC50 (9.2 μM) values were calculated using the exponential equation derived from the data and refer to the concentrations of 2,4-DCP needed to reduce frond reproduction by 10 and 50%, respectively. It is important to note that these values are dependent on the experimental conditions. For example, the vegetative reproduction is proportional to the ratio of plants to 2,4-DCP. If the experimental conditions are the same as those described above, except that the volume of medium is increased to 100 ml, the plants will be in the presence of twice the amount of 2,4-DCP as they would be using 50 ml. Data obtained using these conditions show 2,4-DCP to be more toxic. Conversely, if only the number of plants is increased, 2,4-DCP appears to be less toxic.

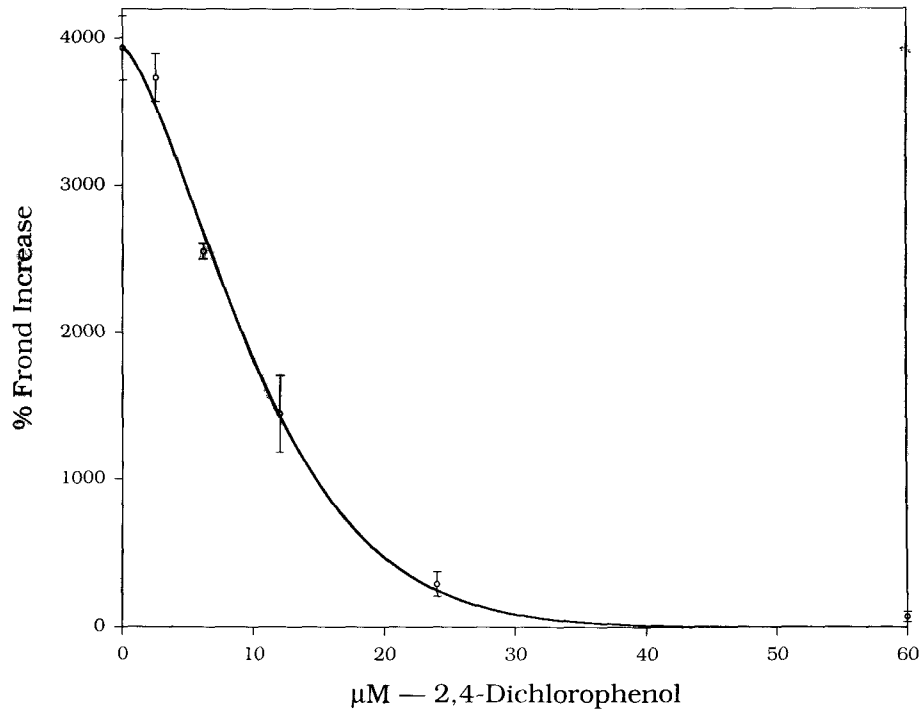

Fig. 1. The effect of 2,4-DCP concentration on the vegetative reproduction of *Lemna gibba* at 27.8°C under continuous light over a 10-d growth period. Each data point is the average of at least 10 replicates, and the error bars represent standard errors.

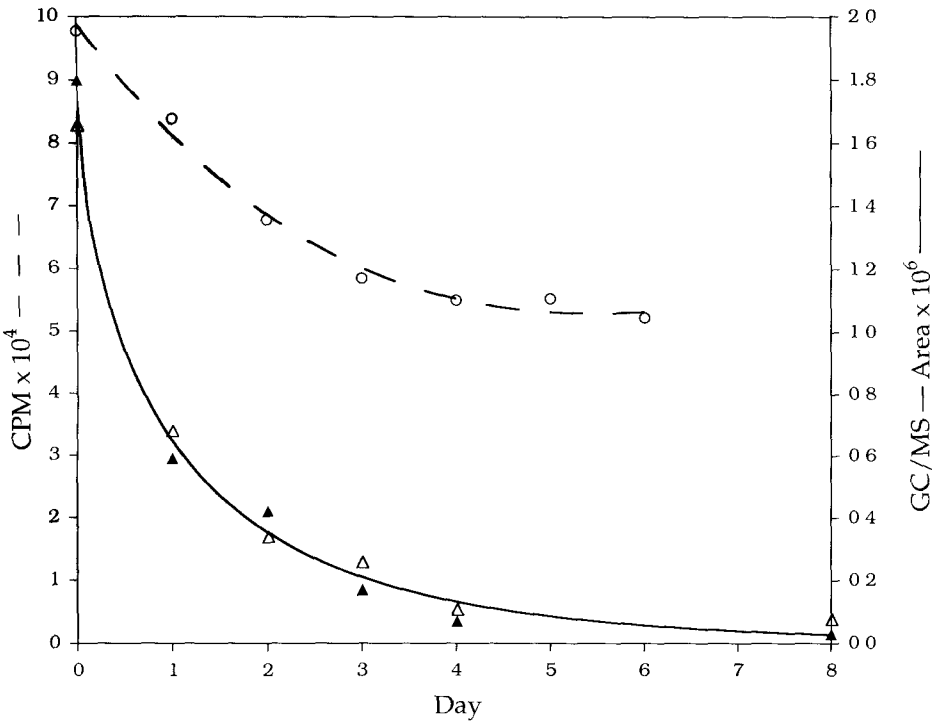

Fig. 2. Analysis of *Lemna gibba* growth medium, which initially contained 15 μM 2,4-DCP (spiked with [U-^{14}C]-2,4-DCP), by selected-ion-monitoring (SIM) GC-MS (solid line) and by scintillation counting (dashed line). Replicate SIM GC-MS data are shown.

SIM GC-MS analysis of control flasks containing medium but no plants showed that there was a small (<10%) loss of 2,4-DCP under the growth conditions employed Therefore, losses of 2,4 DCP >10%, in the presence of plants, can be attributed to plant uptake and/or metabolism Using SIM GC-MS, it appears that the plants removed >90% of the 2,4-DCP by day 6 (see Fig 2, lower curve), and hence, the use of *L gibba* could represent a convenient method to scavenge organic pollutants from an aquatic system However, refer ring to Figure 2 again, the upper curve was generated from the data collected by using scintillation counting, which is a nonspecific monitoring technique This curve shows that there was still approximately half of the original number of counts present in the growth medium The only source of radioactivity was the 2,4 DCP moiety, thus although SIM monitoring of the medium shows that the free phenol is almost completely removed, the 2,4 DCP nucleus is still present, albeit in a modified form

Whereas the results discussed above indicated that duckweed was able to remove approximately 90% of the 2,4 DCP in 6 d, a similar experiment in which the growth medium of *Lemna*, spiked with labeled 2,4-DCP, was analyzed by HPLC showed that after 6 d approximately 66% of the 2,4-DCP had been removed (see Fig 3) There is therefore a certain amount of variability in terms of the rate at which the conversion of 2,4-DCP to metabolite takes place Figure 3 shows the disappearance of the 2,4-DCP (fraction 19), the appearance of the major metabolite (fraction 9), and the appearance of an even more polar metabolite (fraction 4)

The ^{1}H NMR spectrum of the metabolite (Fig 4) is consistent with its being a glucoside, in particular, a β-glucoside The doublet at δ 5 1 is diagnostic of an anomeric proton, and the coupling constant of 7 3 Hz shows that it is a β-glucoside An α-glucoside would have a J value of about 4 Hz [19,20] The spectrum of the synthesized glucoside corresponded to that of the natural compound

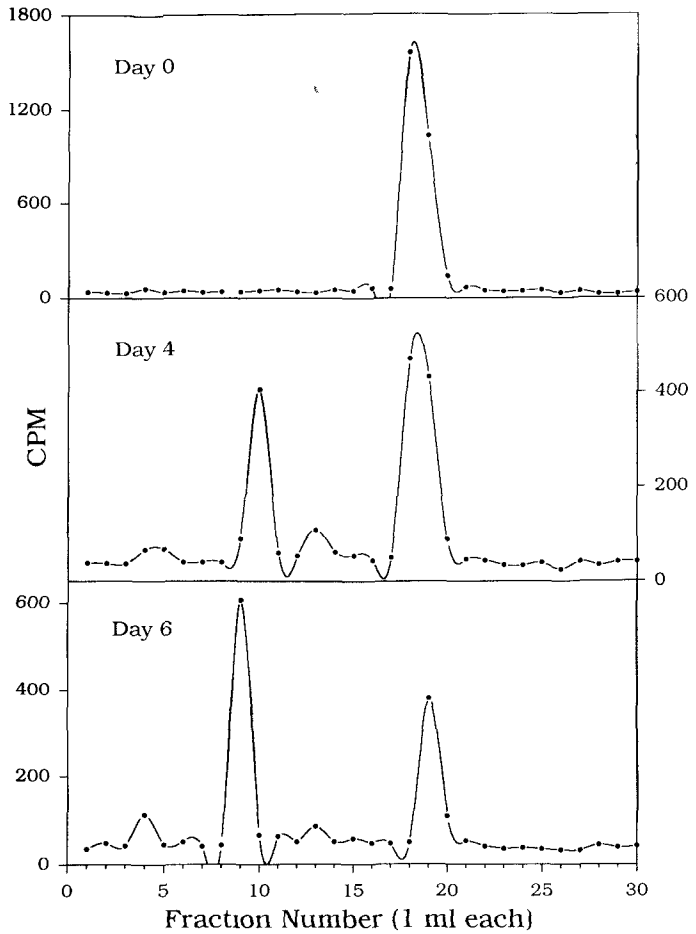

Fig 3 Reversed phase HPLC analyses over time, of *Lemna gibba* growth medium, which initially contained 15 μM 2,4 DCP (spiked with [U ^{14}C] 2 4 DCP) The plants were grown under continuous light at 27 8°C The HPLC elution gradient ran over 30 min, starting with a mixture of 10% acetonitrile 90% water, and ending with a mixture of 99% acetonitrile 1% water One milliliter fractions were collected and counted The peak at fraction 19 is the original 2,4 DCP, that at fraction 9 is the major metabolite, and that at fraction 4 is a very polar secondary metabolite

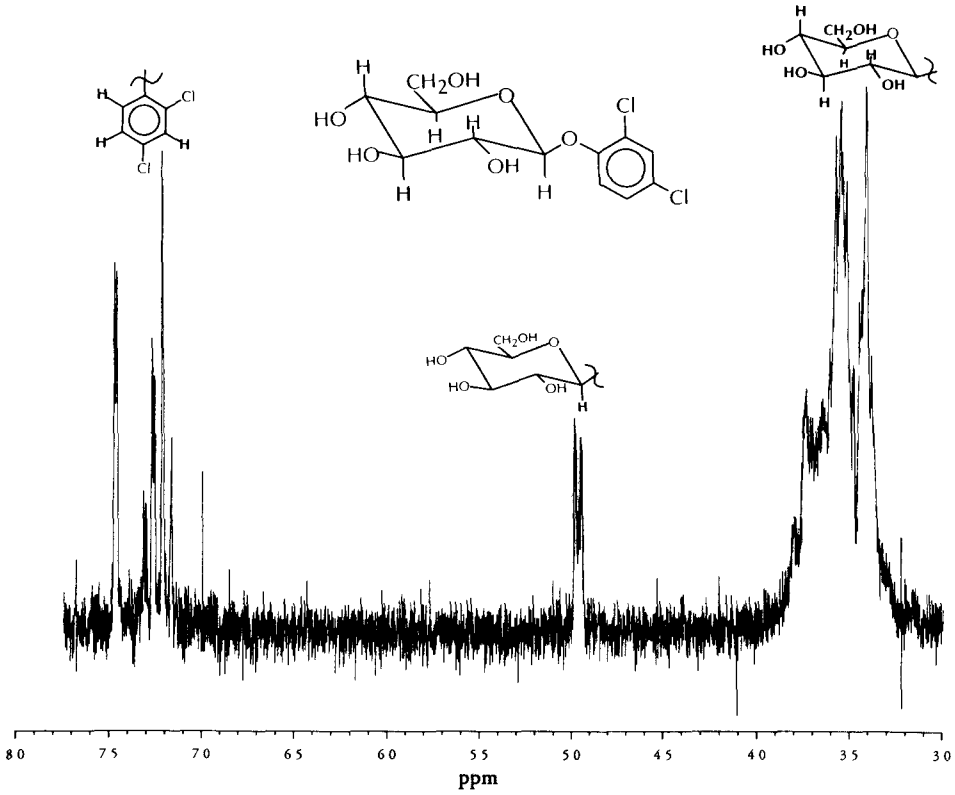

Fig 4 200 MHz ^{1}H NMR spectrum of the major metabolite of 2,4 DCP The aromatic protons appear as a multiplet at 7 1 to 7 5 ppm, the anomeric proton appears as a doublet at 4 9 to 5 0 ppm, and the remaining protons appear as a complex multiplet between 3 3 and 3 9 ppm The structure suggested by this spectrum is shown at center top

The synthesized and natural metabolites were silylated and analyzed by GC MS Both compounds had the same GC re tention time and *m/z* values of 614 (M^+), 597 ($M - CH_3$)$^+$, 507 ($M - ((CH_3)_3SiOH + CH_3)$)$^+$, and 451 ($M - 2,4$-DCP)$^+$

As further evidence that the natural metabolite was the β-glucoside, it was treated with β glucosidase and the prod ucts were analyzed by RP HPLC and scintillation counting The analysis showed that the metabolite was no longer present and that free 2,4-DCP had appeared (Fig 5)

Metabolic studies of the chlorinated phenols include a comprehensive analysis of the degradation of 2,4-DCP by the wood rotting fungus, *P chrysosporium* [10] The fungus uses peroxidases to oxidatively dechlorinate 2,4 DCP to 2,5-dimethoxy-1,4 benzoquinone Through a complicated multistep pathway, the benzoquinone is reduced to 1,2,4,5-tetrahydroxybenzene The aromatic ring of the tetrahydroxy benzene is then cleaved to yield malonic acid, and ultimately CO_2 Higher plants respond differently to chemical challenge by 2,4 DCP, our finding that *L gibba* conjugates it with β D-glucose is the first such report for this plant but agrees with observations that other higher plants metabolize xenobiotics by transformation, conjugation, or compartmen tation [11,14,21] The occurrence of β D-glucosides has been documented, along with the enzymes that catalyze their for mation [11,21] Although we have no quantitative data regarding the relative toxicity of the metabolite vs the parent phenol, it is evidently lower, because the growth medium becomes less toxic to the duckweed as the phenol is metabolized The significance of conjugation to the plant is that it converts one compound to another that is less toxic and more easily expelled The problem of phenol contamination has not been mitigated but instead has been changed to a problem of 2,4 dichlorophenyl β D-glucopyranoside contamination

CONCLUSIONS

The effect of 2,4 DCP on the vegetative reproduction of *L gibba* has been studied EC10 and EC50 values of 2 5 and 9 2 μM, respectively, were calculated using the exponential equation derived from the growth data We have shown that the plants take in the phenol and return it to the growth medium as a β-glucoside The toxicity of the glucoside to various organisms has yet to be determined, but it is apparent that the glucoside can act as a source for the 2,4-DCP moiety Selective ion monitoring of the growth medium using GC MS is not sufficient to determine the presence of such pollutants This is an important consideration for those who monitor pollutants in aquatic ecosystems The plants in those systems, and possibly other organisms, may simply mask the

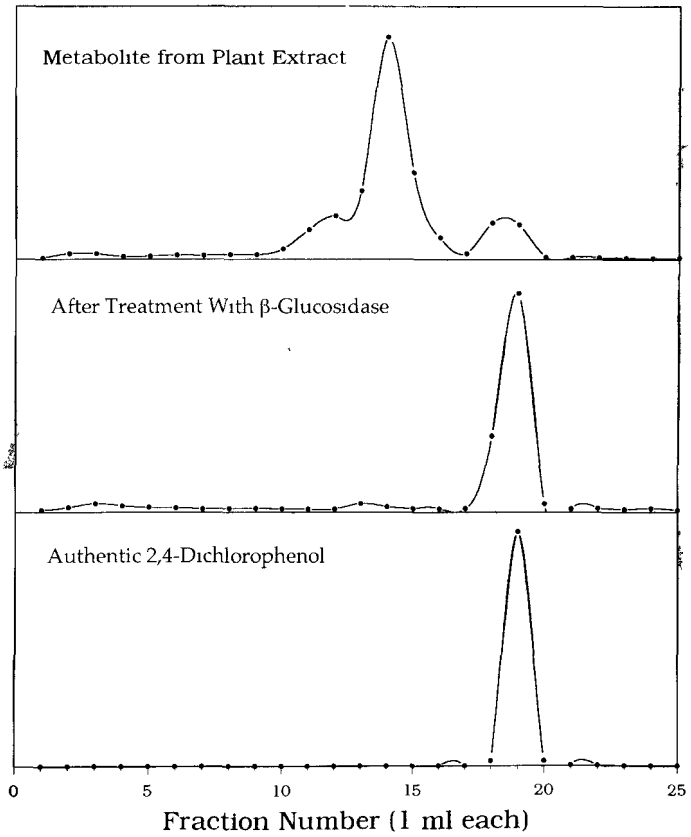

Fig. 5. Reversed-phase HPLC of the radiolabeled major metabolite of 2,4-DCP (fraction 14) before (top) and after (middle) treatment with β-glucosidase. Authentic 2,4-DCP (fraction 19) is shown at the botom. The HPLC analyses used a 25-min gradient, starting with a mixture of 15% acetonitrile:85% water, and ending with a mixture of 30% acetonitrile:70% water. One-milliliter fractions were collected and counted.

presence of certain chemicals by conjugation. These conjugates then act as nonpoint sources for the same pollutant that was thought to have vanished.

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