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Effects and metabolism of the phenylurea herbicide isoproturon in the submerged macrophyte *Ceratophyllum demersum* L.

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Summary

Phenylurea herbicides such as isoproturon (IPU) restrain photosynthesis by connection to the D1 protein in the photosynthetic apparatus in target plants such as weeds in crop fields. Direct effects of herbicides on organisms, which are not a target of the pesticide, have been examined seldom. Since a many of agriculturally used pesticides are found in surface waters in agricultural areas, we determined the effects on the photosynthetic oxygen production of the submerged macrophyte *Ceratophyllum demersum* using concentrations of IPU ranging from 0.2 µg/L to 200 µg/L IPU. At environmental relevant concentrations of IPU, the photosynthetic oxygen release was impaired. A reduction of the photosynthetic oxygen release showed a time dependency with the assigned herbicide concentrations. Furthermore, this study presents the first indications for metabolism of IPU in the aquatic plant *C. demersum*.

Introduction

The environment encounters an increasing number of chemicals of anthropogenic origin. Similar to animals, plants are also able to metabolize and biotransform a great variety of xenobiotics. Research on such mechanisms has mainly focused on terrestrial plants, but those plants seem not to be as sensitive as aquatic plants (GARTEN and FRANK, 1984). The submerged freshwater macrophyte *Ceratophyllum demersum* L., which is also called coontail, is one of the most widespread species in European lakes and rivers and can thus represent a high macrophyte biomass. Due to the abundance of this higher aquatic plant, it can play a major role in detoxifying agricultural xenobiotics but detoxification increases energy demands and consequently plant reproduction may be impaired.

One of the most extensively used pesticides in conventional European agriculture is the phenylurea herbicide isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea (IPU)] (FEDERAL ENVIRONMENTAL PROTECTION AGENCY, 2000). IPU is used for pre- and postemergence control of annual grasses as well as broad leave weeds in wheat, rye and barley crops. Each year approximately 30 t of pesticides reach the surface water in Germany with IPU contributing 2 t annually. As a result of its extensive use and its properties of moderate persistence and relatively low adsorption, IPU is detected in ground- and surface waters in Europe in levels which exceed the European Commission drinking water limit of 0.1 µg L^{-1} (SPLIID and KØPPEN, 1998). Although information about effects of herbicides on terrestrial and aquatic organisms is increasing, there is little known about effects of IPU on freshwater macrophytes. Few studies focused on rooted macrophytes like *Elodea densa* and *Ludwigia natans* (GROUSELLE et al., 1995; FEURTET-MAZEL et al, 1996). RANA and KUMAR (1995) showed that *Elodea densa* is far more sensitive to high doses of IPU than *Ipomoea aquatica* and the composition of phytoplankton was changed by exposure to this herbicide. From these studies it is apparent that aquatic plants show different sensitivity to herbicides like IPU due to their differences in morphology and metabolism. IPU is known to be a selective systemic herbicide, absorbed by the roots, and rapidly transported through the xylem to the leaves. The mode of action is the inhibition of the photosynthetic electron transport (BERGER and HEITEFUSS, 1991). In the macrophyte *Elodea densa* GROUSELLE et al. (1995) showed, that the main binding site for IPU is represented by the D1 protein of the photosynthetic apparatus.

The bioconcentration of IPU in aquatic plants exposed to this herbicide ranges from 100 to 1,200 depending on the initial IPU concentration and is higher than usually calculated by numerical models (CRUM et al., 1999; MERLIN et al., 2001). Biotransformation of IPU in aquatic organisms is possible as shown for some bacteria (SØRENSEN and AAMAND, 2001). Additionally, ecotoxicological data showed that IPU and some of its metabolites could be harmful to aquatic organisms like amphibians (GREULICH et al., 2002). The first phase of metabolism of IPU involves hydroxylation of carbon atoms to create reactive functional groups and is catalyzed by cytochrome P450-dependent monooxygenases (GLÄSSGEN et al., 1999). In a second step, these activated electrophilic derivatives can be conjugated to glutathione, catalyzed by glutathione-S-transferase (GST) in Phase II (GEORGE, 1994). The hydrophilic products of conjugation can be transported into cell compartments or cell walls in the third phase of biotransformation. GST is widely distributed in organisms and may be implicated in cell-line resistance to pesticides (HAYES and WOLF, 1988; GLÄSSGEN et al., 1999). Biotransformation of aquatic contaminants by conjugation to glutathione is well documented and especially the biotransformation of herbicides is often followed by conjugation to this molecule (LAMOUREUX and RUSNESS, 1989).

In the present study, we describe effects of IPU on the photosynthetic oxygen production and the metabolism of this herbicide in *Ceratophyllum demersum* to evaluate the fate of IPU which represents the first step for environmental risk assessment.

Material and methods

Plant material

Ceratophyllum demersum was collected during the summer season from different sites around Berlin. Identification of the plant species was done according to CASPER and KRAUSCH (1980). Plants were cultivated continuously non axenically prior to the experiment for three years in distilled water containing 0.09 g/L $CaCl_2$, 0.03 g/L $NaHCO_3$ (Merck; Darmstadt, Germany) and 0.03 g/L of commercial seasalt (Sera GmbH; Heinsberg, Germany), and in 100 L tanks. Supplementary light was provided by daylight lamps at a light / dark cycle of 12 : 12 h. Temperature was maintained at 22 - 24 °C. Only young shoots were used for the experiments.

Measurement of photosynthetic oxygen production

One gram fresh weight (FW) of the coontail was exposed to 1000 ml medium containing different concentrations (0.2, 2, 20 and 200 µg/L) of isoproturon (Riedel-de Haen; Seelze, Germany). Each exposure was performed in four replicates. In control experiments no isoproturon was applied to the medium. The measurements of photosynthetic oxygen production of the plant were performed using a

Phosy-Mess 4000 (INNO Concept; Straussberg, Germany), 100 % light intensity (SI Unit: 2000 lx) and a dark/light/dark cycle of 10/12/10 min under constant temperature of 20 °C. Measurements were taken with a Clark electrode (WTW EO 196-1,5; Sensortechnik Meinsberg GmbH, Meinsberg, Germany). The rates were calculated in mg O_2 * h^{-1}* g FW^{-1}.

Extraction of isoproturon metabolites and analysis by HPLC

One gram of coontail was exposed to 2000 ml medium containing 25 µg/L IPU. After 24 hours the plant was rinsed thoroughly and frozen in liquid nitrogen and stored under -80 °C until extraction of metabolites. In order to follow the fate of IPU in the surrounding medium 250 ml of the exposure solution were applied onto solid-phase cartridges (Sep-Pak Plus tC18 Environ. Cartridges, Waters, Milford, Massachusetts, US) equilibrated with 5 ml methanol followed by 5 ml sodium phosphate buffer (0.1 M, pH = 6.5) to enrich the target compounds. The sorbent was eluted with 1 ml of methanol and the IPU metabolites analysed by HPLC.

The frozen plant material was grounded using a mortar and liquid nitrogen. The frozen plant powder was added slowly to 10 ml of 70 % methanol and stirred for 30 minutes on ice. The solid cell material was separated by centrifugation (12,100 x g, 5 minutes). The supernatant was analysed by HPLC directly to reduce the possibility of produced oxidation artifacts.

Analyses of IPU and its metabolites were performed using a liquid chromatograph (Waters, Milford, Massachusetts, US) with an injection valve with a 100-µl loop. IPU and its metabolites were seperated using a LiChrospher 100 RP-18 column (250 x 4 mm, 5 µm; Merck, Darmstadt, Germany) and a guard column of the same material (4 x 4 mm). The separation took place at 40 °C at a flow rate of 1 mL/min. The 996 diode array UV detector was set at 240 nm. The separation was performed with a liquid gradient consisting of water and acetonitrile in a composition as described by HAAS (1997). The run of one sample took 65 minutes.

HPLC and electron spray injection – mass spectrometry

Reverse-phase HPLC separations were carried out on the plant extract according to KERTSCHER et al. (1998) on a Vydac C_{18} column (150 x 1 mm I.D., 5 µm, Type 218TP5115), using a dual syringe pump as solvent delivery system (Applied Biosystems, 140B, mixing chamber 60 µL). The injection volume was 100 µL. Mobile phases A and B consisted of water, 0.06% TFA, and acetonitrile-water (8:2, v/v), 0.05% TFA, respectively. Runs were performed at a linear gradient of 8 to 60% B in 40 min and an eluent flow rate of 30 µL/min. The total flow was divided at the column outlet resulting in the introduction of 4 µL/min in a UV detector (785A, Applied Biosystems) with micro cell type ZU (LC Packings), λ = 220 nm, and 26 µL/min *via* a fused-silica capillary directly to the electron spray interface. Mass spectrometry was performed on a triple quadrupole instrument (TSQ 700, Finnigan MAT, Bremen, Germany) equipped with an electron spray ion source (API-ESI) operating in the positive mode and with a capillary temperature of 200 °C and a high voltage of 4.5 kV. Nitrogen was introduced as sheath gas (3.4 bar) and auxiliary gas (960 mL/min). Isopropanol-propionic acid was applied as sheath liquid (25:75, v/v; 4 µL/min).

Statistics

Analysis of significance of the photosynthetic oxygen production between control and exposures was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls test (SPSS 9.0 for Windows).

Results

Photosynthetic oxygen production

As shown in Fig. 1, there is a time-dependent reduction of the photosynthetic oxygen production in *C. demersum* after exposure to all four concentrations of IPU compared to control measurements. Photosynthetic oxygen production was significantly ($p = 0.05$) inhibited after 48 hours exposure to 0.2 µg/L IPU compared to control. Although the exposure to 2 µg/L IPU for 48 hours caused a reduction of the photosynthetic oxygen release of 54 % compared to control, but this decrease was not significant. A 66 % reduction of the photosynthetic oxygen production compared to control was observed after 48 h-exposure to 20 µg/L IPU. Measurements of photosynthetic oxygen production in *C. demersum* after treatment with the highest IPU concentration (200 µg/L) for 48 hours showed a significant decrease of 70 % when compared to control.

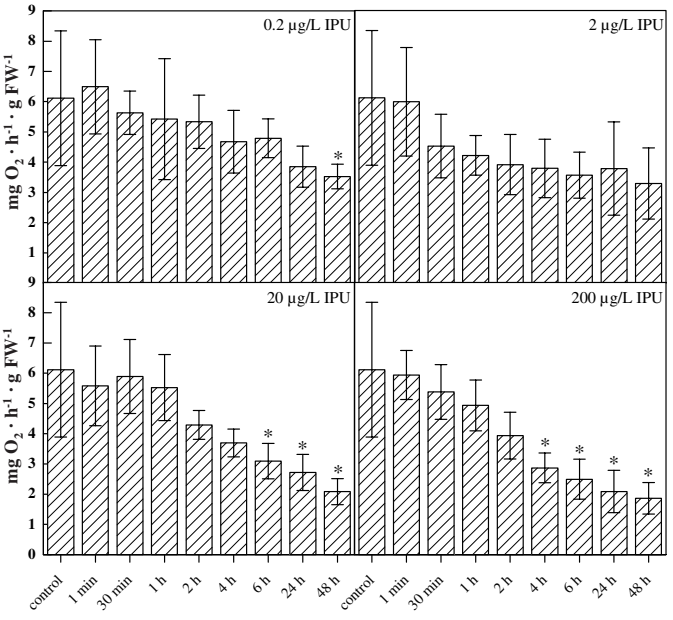

Fig. 1: Time-dependent reduction of photosynthetic oxygen release of *C. demersum* after exposure to the four concentrations of IPU (0.2; 2; 20 and 200 µg/L).

HPLC analysis

HPLC analysis of the medium after exposure to 25 µg/L isoproturon (Fig. 2B) showed that *C. demersum* is able to metabolize IPU to quite a large amount of metabolites indicated by distinct peaks with lower retention times than IPU. In the plant extract no peak was detectable at the retention time of pure IPU (26 min, Fig. 2A).

Mass spectrometry

Analysis of the *in-vivo* extract from the coontail by mass spectrometry showed that *Ceratophyllum demersum* is able to transform the herbicide IPU (m/z = 207.0) to a variety of hydroxylated, demethylated and conjugated metabolites. Our measurements showed the metabolites didesmethyl-IPU and monodesmethyl-IPU identified as signals at masses of 176.0 and 193.0 (m/z), respectively (Fig. 3A). The metabolite isopropenyl-IPU occurred at a mass of 205.1 (m/z). Metabolite OH-isopropyl-IPU exhibited a protonated ion at m/z 223.0 (Tab. 1). A glutathione metabolite of OH-isopropyl-IPU was detec-

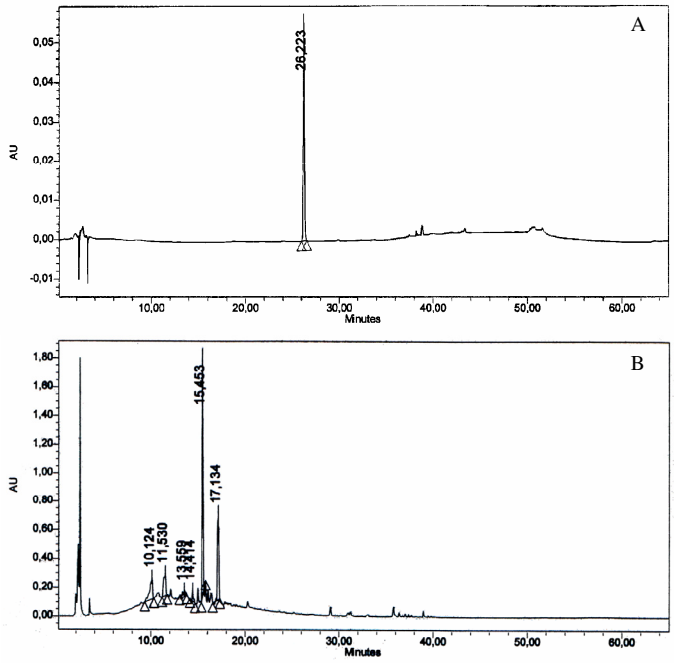

Fig. 2: HPLC elution profiles of isoproturon (A) and its metabolites in the *in-vivo* extract (B) from *Ceratophyllum demersum* detected at 240 nm with reference to the elution time.

ted at a mass of 527.1 (m/z) (Fig. 3B). As a degraded glutathione conjugate of the same metabolite occurred cysteine-OH-isopropyl-IPU at m/z of 341.0 (Tab. 1).

Different derivates of monodesmethyl-IPU were detected. A very distinct peak showed the conjugate of cysteine and monodesmethyl-IPU at m/z 543.1 (Fig. 3B). The metabolite hydroxy-monodesmethyl-IPU showed a protonated peak at 208.9 mass units (Fig. 3A). The degraded glutathione conjugates of the same molecule were found as a defined peak at a mass of 327.1 (m/z), which was identified as the conjugate of cysteine and OH-monodesmethyl-IPU (Tab.1). The peaks at the masses of 404.8 and 294.0 (m/z) corresponded to the degraded glutathione conjugates of didesmethyl-IPU (γ-glutamyl-cysteine-didesmethyl-IPU and cysteine-didesmethyl-IPU, respectively).

Based on the mass spectrometry of the IPU metabolites, Fig. 4 represents a possible scheme for the metabolization of IPU in the freshwater macrophyte *C. demersum*.

Discussion

Photosynthetic oxygen production

Herbicides control a broad spectrum of weeds without affecting the crops. But action of pesticides in agriculture is not confined to the target organisms alone and it is necessary to evaluate their effects on aquatic ecosystem. Because of its common use IPU is widely distributed in the environment. Concentrations in a French river surface water reached up to 2.6 µg/L (IRACE-GUIGAND et al., 2004). KIRBY and SHEAHAN (1994) reported short-lived values in agricultural run-off waters occurred concentrations up to 17 µg/L IPU and a maximum concentration of 500 µg/L of this herbicide was noted for drain water from a crop field after rainfall event by JOHNSON et al. (1996). Consequently, the concentrations used in this study are environmentally relevant.

Tab. 1: Apparent metabolites in the in-vivo extract from *C. demersum* after exposure to 25 µg/L IPU analysed by ESI-TOF.

mass peak	metabolite
176.0	didesmethyl-IPU
193.0	monodesmethyl-IPU
195.0	OH-didesmethyl-IPU
205.1	isopropenyl-IPU
207.0	IPU
208.9	OH-monodesmethyl-IPU
223.0	OH-isopropyl-IPU
294.0	cysteine-didesmethyl-IPU
327.1	cysteine-OH-monodesmethyl-IPU
341.0	cysteine-isopropyl-IPU
404.8	γ-glutamyl-cysteine-didesmethyl-IPU
527.1	OH-isopropyl-IPU
543.1	cysteine-monodesmethyl-IPU

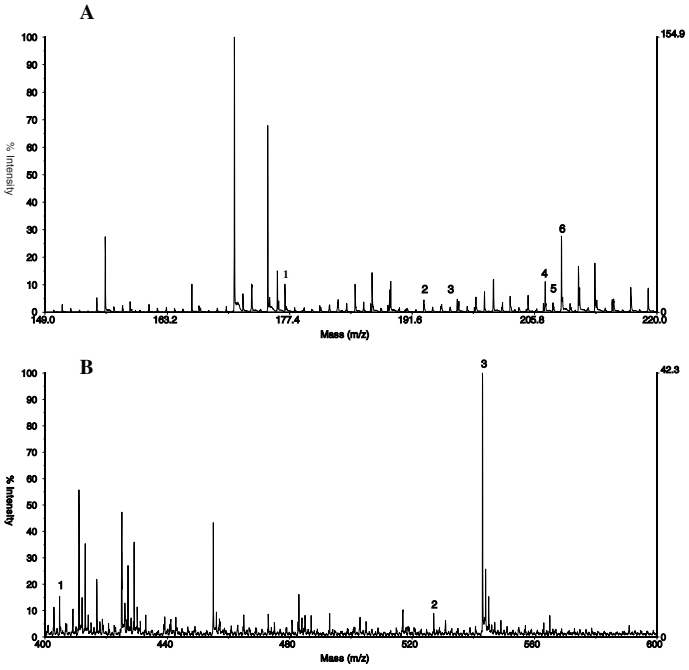

Fig. 3: Chosen ESI spectra (150-220 and 400-600 m/z) of *in-vivo* extract from *C. demersum* after exposure to 25 µg/L IPU for 24 h. m/z : **A: 1** = didesmethyl-IPU(176.0); **2** = monodesmethyl-IPU (193.0); **3** = OH-didesmethyl-IPU (195.0); **4** = isopropenyl-IPU (205.1); **5** = IPU (207.0); **6** = OH-monodesmethyl-IPU (208.9); **B: 1** = γ-glutamyl-cysteine-didesmethyl-IPU (404.8); **2** = OH-isopropyl-IPU (527.1); **3** = cysteine-monodesmethyl-IPU (543.1).

For photosynthesis in plants is considered as a process sensitive to environmentally changes and IPU is acting as an inhibitor of the photo-dependent electron transport at the photosystem II level, effects on metabolism and growth of plants are the consequence (BERGER and HEITEFUSS, 1991). Despite the non-significant reduction of the photosynthetic oxygen release after 48 h exposure of coontail to 2 µg/L IPU, the lower concentrations of isoproturon (0.2 and 2 µg/L) already had a pronounced effect on this important metabolic func-

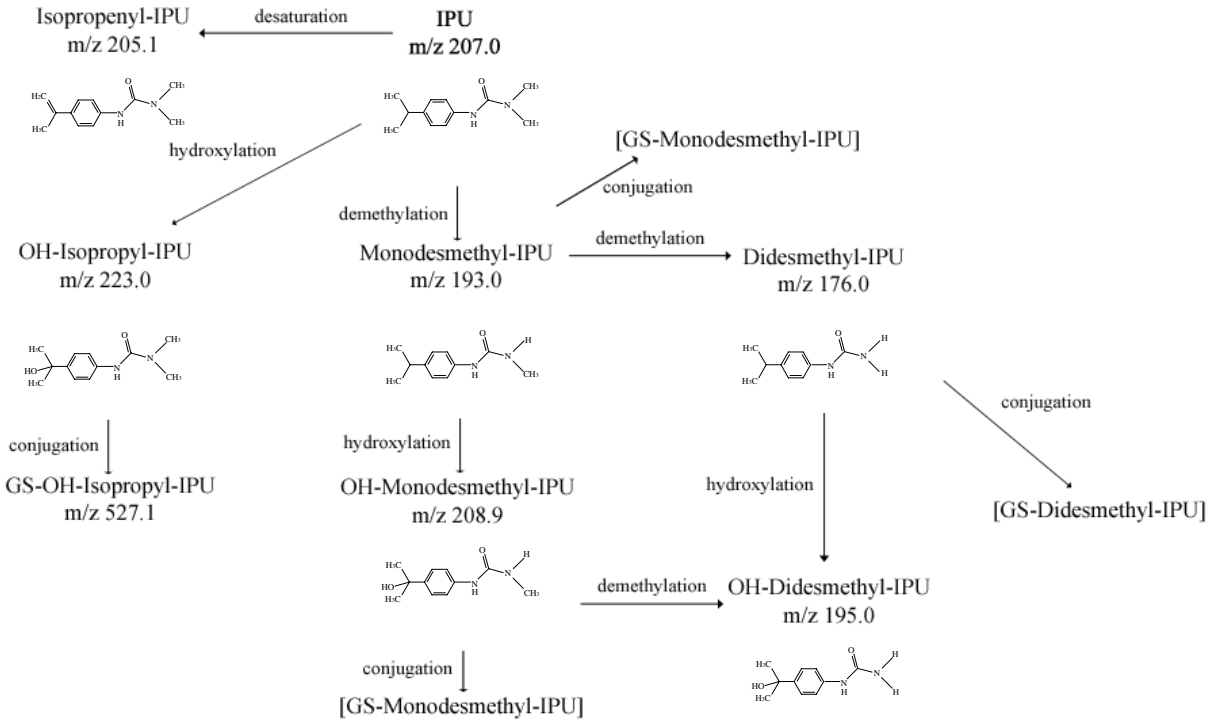

Fig. 4: Metabolic scheme for isoproturon in *Ceratophyllum demersum* based on the ESI-TOF elution profiles at 240 nm. The protonized masses (m/z) and abbreviated trivial names as used in the present article are indicated together with the metabolic structures. Metabolites in brackets were not detectable in *C. demersum* but are possible short-lived intermediates.

tion in *C. demersum* in this study. After exposure to 20 µg/L IPU the negative effect on photosynthesis was even stronger and the photosynthetic oxygen release was inhibited significantly beyond an exposure time of 2 hours to 200 µg/L IPU. This is in agreement with the observations of FEURTET-MAZEL et al. (1996), showing that the dissolved oxygen concentration in the surrounding medium decreased due to inhibition of photosynthesis in *Elodea densa*.

Damage to aquatic organisms by pesticides is known to be related to their potential toxicity and their duration of exposure. Additionally, age of plant shoots seems to be of importance as shown by WEINBERGER and GREENHALGH (1985) for absorption of the pesticide aminocarb to *Ceratophyllum demersum.* As a consequence, we used young shoots of coontail only.

GROUSELLE et al. (1995) showed that the photosynthetic inhibition in plants by IPU is mainly due to specific binding to the D1 protein, a 32-kDa protein of the photosystem II within the thylakoid membranes. IPU causes a rapid bioaccumulation in aquatic macrophytes (CRUM et al., 1999). High bioconcentration factors for aquatic macrophytes indicate that low IPU concentrations show a clear saturation tendency in these plants (FEURTET-MAZEL et al., 1996). This saturation effect is caused by the specific binding of IPU to the D1 protein, while exposure to higher IPU concentrations results in lower bioconcentration factors in *Elodea densa* due to non-specific binding of IPU (GROUSELLE et al., 1995). This may be caused by the lipophilic properties of IPU resulting in an higher toxicity to macrophytes than to algae (KIRBY and SHEAHAN, 1994). A concentration of 10 µg/L IPU caused significant negative effects on the growth of *Elodea densa*, and concentrations higher than 200 µg/L IPU were followed by a total growth inhibition (FEURTET-MAZEL et al., 1996). Furthermore binding of IPU to D1 protein in algae and higher plants results in a breakdown of the photosynthetic synthesis of adenosine triphosphate and nicotinamide adenine dinucleotide phosphate and thus further impact on metabolism can be expected.

HPLC analysis

After an exposure of 24 hours to IPU (25 µg/L), the parent compound is no longer detected in the medium, but quite a variety of herbicide metabolites were observed by HPLC. This indicates that metabolization of IPU by this macrophyte is rather fast as already shown by CRUM et al. (1999). Depending on weather conditions IPU might be more persistent in soil than indicated by its half-life (JOHNSON et al., 1996), and even if farmers follow good agricultural practice, herbicides applied to winter cereals might reach nearby ditches and streams and thus contaminate surface waters. The average half-life of IPU in the water column ranged from approximately 50 to 60 days (PERES et al., 1996). COOPER et al. (2004) showed that vegetated agricultural ditches affect the mitigation of pesticides. Vegetation in ditches plays an important role in the transformation of contaminants and can thus be an environmental benefit. Macrophytes accelerate the IPU removal from the water column while its adsorption to sediment and photochemical degradation play a negligible role (MERLIN et al., 2001; AMINE-KHODJA et al., 2004). Additionally, buffer zones between field crops and ditch banks can reduce pesticide deposition on ditches.

Though the presence of macrophytes may result in lower environmental pesticide concentrations the accumulated contaminants can also be a long-term source for aquatic contaminants and thus further threaten organisms living in or off aquatic plants. According to MERLIN et al. (2001) aquatic macrophytes are able to accumulate approximately 45 % of the IPU applied. Average bioconcentration factors for IPU, calculated by GROLLIER et al. (1996), were close to 10 in *Elodea densa* and *Ludwigia natans*. The rapid metabolization of IPU in our experiments also revealed the shift to derivatives with lower retention time indicating the production of more hydrophilic metabolites. Because reference standards for IPU metabolites are rare, further characterization of metabolites by mass spectrometry was applied.

Mass spectrometry

Methods like Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) are not being employed very often in plant metabolic studies, but our results are in agreement to those by GLÄSSGEN et al. (1999), who showed the metabolization of IPU in wheat and soybean cell cultures was mainly by hydroxylation and dealkylation. These reactions are catalysed by cytochrome P450 enzymes. A ring-methyl hydroxylation of IPU was shown in wheat cell cultures (MOUGIN et al., 1991). In *C. demersum* we also found the hydroxylated derivative isopropyl-IPU. ROBINEAU et al. (1998) reported that the monooxygenase CYP76B1 in *Helianthus tuberosus* actively metabolizes phenylureas via a double N-dealkylation. In this plant the herbicide IPU is mainly metabolized to mono- and didealkylated derivatives with the second demethylation being the rate limiting step of detoxification. The dealkylated derivatives of IPU identified in this study were monodesmethyl-IPU and didesmethyl-IPU which are both further processed to hydroxylated metabolites. The generation of OH-didesmethyl-IPU might be possible via demethylation of the monodealkylated IPU derivative and followed by hydroxylation. On the other hand the detoxication of monodesmethyl-IPU via hydroxylation to OH-monodesmethyl-IPU and further processing to OH-didesmethyl-IPU might be more important in *C. demersum*. The existence of the desaturated metabolite isopropenyl-IPU is in agreement to the observations of GLÄSSGEN et al. (1999) though its importance remains unknown. In summary the tolerance and selectivity of phenylureas in plants seems to rely on the presence and relative expression of at least two P450 enzymes. Further research is needed to determine the importance of the different possible detoxication pathways.

The second step of biotransformation consists of the conjugation of xenobiotics to glutathione (GSH) or other cell-internal molecules. In our study the only glutathione conjugate detectable in coontail is glutathione-hydroxy-isopropyl-IPU (GS-OH-isopropyl-IPU), but the amount of cysteine conjugates and γ-glutamyl derivatives indicates that the majority of the produced glutathione conjugates is degraded rapidly. The conjugation of monodesmethyl-IPU to glutathione seems to be of importance as indicated by the pronounced peak of the cysteine derivative of this metabolite on the ESI spectrum.

It is also known that herbicides can be activated by biotransformation. Though TRIXIER et al. (2001) showed that hydroxylation of phenylureas leads to non-toxic derivatives, demethylation can generate products of high toxicity. According to MÜLLER (1986), the metabolite monodesmethyl-IPU can be still phytotoxic. GLÄSSGEN et al. (1999) showed that the metabolism of IPU in monocotyledon plants is different from those in dicotyledonous plants. The same author showed that cell culture suspensions of the monocotyledonous wheat produced not as much of the metabolite monodesmethyl-IPU as did cells of the dicotyledonous soybean. Meanwhile the wheat cells seemed not to be affected by IPU as much as soybean cells. Thus the different metabolism of IPU in monocotyledonous and dicotyledonous plants may be the reason for differences in sensitivity to this herbicide. For IPU seems to be reacting selectively on dicotyledonous plants, this might explain the pronounced impact on the cormophyte *C. demersum*.

In conclusion, this study shows a great impact of IPU on photosynthetic oxygen production of the aquatic macrophyte *C. demersum*. Furthermore the metabolization of IPU by *C. demersum* showed that this plant behaves like a typical dicotyledonous plant and this can partially explain its sensitivity to IPU.

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