The fate of the herbicide propanil in plants of the littoral zone of the Three Gorges Reservoir (TGR), China

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ABSTRACT

The anti-seasonal hydrology with 30 m water fluctuations in the Three Gorges Reservoir (TGR) of China attracts growing environmental and ecological concerns. We investigated the biotransformation of the herbicide propanil in plants dominating in the littoral zone of the TGR by applying the ^{14}C-ring-labeled herbicide into non-aseptic hydroponic plant systems (*Cynodon dactylon*, *Nelumbo nucifera* and *Bidens pilosa*), aseptic plants (*Lemna minor* and *Lemna gibba*) and cell suspension cultures (*C. dactylon* and *L. minor*). (1) Propanil absorbed in plants of the hydroponic systems was $(12.46 \pm 1.63)\%$ of applied radioactivity (AR) (*C. dactylon*), $(52.36 \pm 6.38)\%$ (*N. nucifera*) and $(76.55 \pm 6.07)\%$ (*B. pilosa*), respectively. The ^{14}C-residues in the plant extractable fractions and the corresponding media were confirmed by radio-Thin Layer Chromatography (TLC), radio-High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Electron Ionization Mass Spectrometry (GC–EIMS) as propanil, 3,4-dichloroaniline (DCA) and N-(3,4-dichlorophenyl)-β-D-glucopyranosylamine (Glu-DCA). (2) About 8% of AR was taken up by both aseptic plants, from which 7.0% of AR was extracted and identified also as propanil, DCA and Glu-DCA. (3) Concerning cell suspension cultures, $(39.22 \pm 9.39)\%$ of AR was absorbed by *C. dactylon* after 72 hr, whereas the accumulated ^{14}C-propanil by *L. minor* cell suspension culture amounted to $(65.04 \pm 1.72)\%$ after 7 days. The identified compounds in cell cultures are consistent with those in the tested plants. Most of the pesticide residues in the intact plants were un-extractable, which are recognized as the end of the detoxification process. We therefore consider these plants as suitable for the phytoremediation of the herbicide propanil in the TGR region.

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Introduction

The Three Gorges Dam (TGD) was constructed in the Yangtze River (Yichang, China) for the main purposes of irrigation, power generation, navigation and water providing. Since 2008, the dam started in full function and the corresponding Three Gorges Reservoir (TGR) covers an area of 58,000 km² in total (water surface area: 1080 km²; riparian zone: 349 km²). The anti-seasonal hydrology of TGR with water fluctuations of 30 m difference in height (the lowest water level is 145 m above sea level in summer while the highest is 175 m in winter) considerably influences the biogeochemical patterns in the TGR area; thus it attracts the concerns regarding environmental and ecological aspects, *i.e.*, water quality,

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nutrient and pollutant loads, sedimentation, greenhouse gas emission and biodiversity (Li et al., 2013). The decline of plant diversity is a matter of fact, about 55% of vascular flora species disappeared or became rare after the first 30 m height winter flooding (Sun et al., 2012; Wang et al., 2012). Vegetation succession in the littoral zone of the TGR thereby depends mainly on the survival species. In contrast to the above submerged region of TGR (e.g., 175–200 m), higher plant richness and biomass in the littoral zone have been also observed (Sun et al., 2012). Dominant species reflect the characteristics of plant community, thus they are ideal target species for ecological restoration engineering. Attempts on enhancing the environmental quality and biodiversity in the TGR region have been conducted (Willison et al., 2013), in which the cultivated plants not only create habitats for wetland biota but also accumulate nutrients and pollutants from upstream and within the basin. Concerning natural vegetation in the littoral zone of the TGR, little is known regarding pollutant remediation.

Wetlands are the "kidney" of nature as they can degrade pollutants, depending on the macrophytes and the corresponding rhizospheric microbes. The metabolism of xenobiotics in vegetation has been widely investigated via plant cell cultures, hydroponic plants and excised plant parts (Davis et al., 1978). In the past decades, remediation on organic pollutants such as trichloroethylene (TCE) and pesticides have been carried out by terrestrial and wetland species (Newman et al., 1998). In the Midwestern United States, three common aquatic species, Ceratophyllum demersum, Elodea canadensis, and Lemna minor were found to be able to accelerate the degradation of metolachlor and atrazine in herbicide contaminated waters (Rice et al., 1997). As reported by Nohara and Iwakuma (1996), at Lake Kasumigaura where the vegetation was predominated by Nelumbo nucifera Gaertn., maximum pesticide concentrations (fenobucarb, diazinon, iprobenfos and simetryn) in water ranged from 1.1 to 2.4 μg/L and the maximum pesticide residues of simetryn in tissue of N. nucifera amounted to 300 μg/kg (wet weight).

Recently, pesticides were found at detectable levels in the surface water of the TGR (Wolf et al., 2013). The Three Gorges region is a traditional rice culture area in China, thus the use of propanil is an option for local farmers. Propanil is a highly selective post-emergence use herbicide for weed control in rice (Oryza sativa L.), which inhibits photosynthesis (photosystem II) in chloroplasts. Data obtained from previous studies verified that it is degraded to propanoic acid and 3,4-dichloroaniline (DCA); the latter is further metabolized, yielding both azo-products such as 3,3′,4,4′-tetrachloroazobenzene (TCAB), 3,3′,4,4′-tetrachloroazoxybenzene (TCOAB) (in soil and sediment) and sugar conjugates (in plants). The acute toxicity of the priority pollutant DCA is higher than that of the parent compound propanil for aquatic organisms such as Daphnia magna; the azo-products, formed as secondary metabolites from DCA are embryolethal, teratogenic and genotoxic although parts of the data are still questionable (Poland et al., 1976; Witt et al., 2000). Besides, considerable portions of DCA and the corresponding metabolites are incorporated into soil organic matter and plant macromolecules (e.g., cellulose, hemicellulose and lignin), forming residues that are hardly or not extracted with various solvents, forming so called non-extractable residues (NER) (Kästner et al., 2014; Schmidt, 1999).

Propanil dissipates rapidly in water-sediment systems with a half-life ranging from 12 hr to a few days (Wauchope et al., 1992), and the amount of DCA in rivers has been found 10 times higher than that of propanil. Therefore, if noticeable amount of these compounds appear in the environment, attention should be drawn to the potential ecological risk (Schmidt, 1999).

The biotransformation of propanil in soils, water and plants (both crops and weeds) demonstrated that plant and soil microorganisms are able to degrade the herbicide (Bartha, 1968; Frear and Still, 1968; Tweedy et al., 1970). Phytoremediation of propanil polluted sites, therefore, is a potential option for natural and artificial vegetation (Williams, 2002). In the present study, we examined the uptake and accumulation of the herbicide propanil in plants dominating in the littoral zone of the TGR. ^{14}C-ring-labeled propanil was applied into different plant and corresponding cell suspension culture systems, and we investigated it's uptake, transport and transformation.

1. Materials and methods

1.1. Chemicals

[Ring-U-^{14}C]-N-(3,4-Dichlorophenyl) propanamide (propanil) was purchased from the Institute of Isotopes (1013 MBq/mmol; radiochemical purity 98.42%). The 3,4-dichloroaniline (DCA) was produced by Riedel-de Haen (purity 99%). Non-labeled propanil (purity 99.6%) and 3,4-dichloroacetanilide (DCAA) were purchased by Sigma. N-(3,4-dichlorophenyl)-β-D-glucopyranosylamine (Glu-DCA) was self-synthesized (Gareis et al., 1992).

1.2. Plant preparation and application

The plants chosen for the study were: Cynodon dactylon, Bidens pilosa, N. nucifera, L. minor and Lemna gibba. C. dactylon is dominating in the littoral zone between 145 m and 160 m around the whole TGR region, whereas B. pilosa appeared in the area above 160 m (Wang et al., 2012). Considering the potential use in environmental conservation of the TGR region (Li et al., 2011), N. nucifera, which can adapt to water level fluctuation, was also chosen. These three dominant species were grown in hydroponic plant systems (non-aseptic). Duckweeds may rapidly grow during flooding events. Both species selected (L. minor and L. gibba) are frequently used in phytotoxicity tests (EPA, 2012). Duckweeds were sterilized and then cultured in the autoclaved nutrient media. Besides, C. dactylon and L. minor cell suspension cultures were established and cultured in the corresponding aseptic media.

1.2.1. Hydroponic plant systems
The plant seeds (C. dactylon) were provided by the company Schwarzenberger Samen & Gartenbedarf (Austria) and cultivated in the greenhouse with commercial soil (Einheits Erde, Type ED73) until 10-leaf stage. The plantlets were pre-washed by Millipore water and then transferred into cultivation flasks (5.0 cm × 8.5 cm, diameter × height) containing 50 mL Hoagland solution. The flasks were kept in the laboratory with illumination (200–300 μmol/(m^2·sec); 16 hr/8 hr light/dark) for 7 days until application.

N. nucifera was supplied from the Botanic garden of Bonn University (Germany) and cultivated in the same greenhouse as *C. dactylon*. The roots were cut from the mother plant and then sub-cultured in glass vessels (8.0 cm × 20 cm), into which ca. 200 g soil and tap water were filled in advance. After growing of new leaves, these plants were washed and transferred into new vessels which contained Hoagland nutrients and sand (Hamann, Germany).

B. pilosa seeds (collected in the TGR area in 2011) were germinated on wet filter-paper in darkness at 22°C. Then the seedlings were transferred onto steel wire gratings (4 mm mesh width) placed upon the surface of cultivation flasks (5.0 cm × 8.5 cm) containing 50 mL of Hoagland solution until the four leaf-stage. Afterwards the plants were individually placed in glass vessels (8.0 cm × 20 cm) containing new fresh Hoagland solution. The vase-cultivating plants were also kept in laboratory with illumination (200–300 μmol/(m^2·sec); 16 hr/8 hr light/dark) until 30 cm height (the nutrient medium was renewed weekly).

Before application, 300 mL fresh Hoagland and 100 mL previous medium (suction filtration, 0.45 μm) were mixed in a vessel, thereby all prepared nutrient solutions contained rhizosphere microbes. ^{14}C-propanil (42 kBq) fortified with the necessary amount of non-^{14}C-labeled propanil was applied to the nutrient medium with the final concentration of 20 mg/L (*C. dactylon*) and 10 mg/L (*B. pilosa* and *N. nucifera*), respectively. Plants were placed into these vessels. Meanwhile, blank controls (vessels without plant) were applied with the same amount of propanil. Both plants and the blank controls comprised each three replicates. Finally, the vessels were individually placed in plastic cases (28 cm × 28 cm × 56 cm), in which two rubber catheters were connected with the case nearby the top (Fig. 1). The airflow thereby can be pumped into the case from one catheter and flows out from the other through an absorption device containing 15 g soda lime for catching [14]CO_2 at the end of the catheter.

1.2.2. Aseptic plants

Axenic *L. minor* was provided in the institute and grown in autoclaved Steinberg media (Steinberg, 1946). The stock solution contained (g/L): S1 (KNO_3 17.5, KH_2PO_4 4.5, K_2HPO_4 0.63); S2 $MgSO_4$·$7H_2O$ 5.0; S3 $Ca(NO_3)_2$·$4H_2O$ 14.75; S4 (H_3BO_3 0.12, $ZnSO_4$·$7H_2O$ 0.18, Na_2MoO_4·$2H_2O$ 0.044, $MnCl_2$·$4H_2O$ 0.18) and S5 ($FeCl_3$·$6H_2O$ 0.76, Na_2EDTA·$2H_2O$ 1.5). The medium (200 mL/flask, S1: 4 mL; S2: 4 mL; S3: 4 mL; S4: 200 μL and S5: 200 μL) was adjusted to pH 5.6 and autoclaved (121°C, 20 min) before usage.

Axenic *L. gibba* was prepared from the stock plant by the following procedure: The stock plants (*L. gibba*) were transferred into a sterilized 500 mL Erlenmeyer flask containing 200 mL autoclaved Millipore water and washed by stirring (500 r/min, 10 min, repeated once). Afterwards, plants were transferred into a new flask: 37.5 mL commercial detergent (Dan Klorix, Germany) were added into the flask and mixed with 200 mL sterilized Millipore water prior to surface sterilization for 10 min. The surface-sterilized plants were cleaned similarly as described above (3 × 10 min) and all processes were performed under sterile conditions. Finally the plants were cultivated in autoclaved 20× APP medium (200 mL/flask) (EPA, 2012), The compounds of the stock

Fig. 1 – Device of the metabolism study in the hydroponic plant systems.

solution (g/L) were: A1 ($NaNO_3$ 26.0, $MgCl_2$·$6H_2O$ 12.0, $CaCl_2$·$2H_2O$ 4.4), A2 ($MgHPO_4$·$7H_2O$ 15.0), A3 (K_2HPO_4·$3H_2O$ 1.4), B1 (H_3BO_3 0.19, $MnCl_2$·$4H_2O$ 0.42, $FeCl_3$·$6H_2O$ 0.16, Na_2EDTA 0.30, $ZnCl_2$ 3.3×10^{-3}, $CoCl_2$·$6H_2O$ 1.4×10^{-3}, Na_2MoO_4·$2H_2O$ 7.3×10^{-3}, $CuCl_2$·$2H_2O$ 0.012×10^{-3}) and C1 ($NaHCO_3$ 15.0).

Axenic plants were sub-cultivated in 300 mL Erlenmeyer flasks (*ca.* 80–100 plants/flask) with 200 mL relevant autoclaved media. Propanil (10 μg, 32 kBq, dissolved in 20 μL methanol) was injected into media (0.05 mg/L) in advance. The flasks were kept in the laboratory under illumination (200–300 μmol/(m^2·sec); 16 hr/8 hr light/dark) and incubated for 12 days. The experiment was performed with triplicates.

1.2.3. Cell suspension cultures

The cell suspensions were cultured in MS media (mmol/L): major inorganic nutrients (A) NH_4NO_3 20.6, KNO_3 18.8, $CaCl_2$·$2H_2O$ 3.0, $MgSO_4$·$7H_2O$ 1.5, KH_2PO_4 1.25; trace elements; (B) KI 5.0×10^{-3}, H_3BO_3 0.1, $MnSO_4$·H_2O 0.01, $ZnSO_4$·$7H_2O$ 0.03, Na_2MoO_4·$2H_2O$ 1.0×10^{-3}, $CuSO_4$·$5H_2O$ 1.0×10^{-4}, $CoCl_2$·$6H_2O$ 1.0×10^{-4}; iron source/Fe-EDTA; (C) $FeSO_4$·$7H_2O$ 0.1, Na_2EDTA·$2H_2O$ 0.1; organic supplement/vitamins; (D) meso-inositol 0.494, nicotinic acid 4.7×10^{-3}, pyridoxine·HCl 2.4×10^{-3}, thiamine·2 HCl 3.0×10^{-4}, glycine 0.03) (Murashige and Skoog, 1962).

L. minor cell suspension culture was prepared from the aseptic plant. Minor portions of plant tissue (3–5 mm) were clipped and placed on the semi-solid medium for callus induction in the dark (27°C). The compounds of semi-solid medium were: MS media (70 mL stock solution per liter medium: A 50 mL, B 5 mL, C 10 mL and D 5 mL), 2,4-dichlorophenoxyacetic acid (2,4-D, 2 mg/mL

stock solution) 6 mg/L, sucrose 30 g/L, agar 6 g/L and then the pH was adjusted to 5.6. After successful callus induction, callus were transferred into 100 mL Erlenmeyer flask (25 mL media/flask; MS media: A 1.25 mL, B 125 μL, C 250 μL and D 125 μL; 2,4-D 12.5 μL; sucrose 0.75 g; pH 5.6) and cultured in the dark with continuous shaking (110 r/min, 27°C).

In order to achieve the callus from *C. dactylon*, the nodes (*ca*. 5–10 mm) from the stock plant were selected and surface sterilized. Nodes were put into a pre-autoclaved 500 mL Erlenmeyer flask containing 200 Millipore water and a rotor, the flask was then placed on a magnetic stirrer (500 r/min) for pre-cleaning (3 × 10 min). Afterwards, the pre-cleaned nodes were surface sterilized in a process mentioned above. Those nodes were washed again several times, in a process similar to the pre-cleaning (3 × 15 min). Finally, the surface sterilized nodes were also cultured in a semi-solid medium containing 1/2 MS media (35 mL stock solution for 1-L medium: A 25 mL, B 2.5 mL, C 5 mL and D 2.5 mL), 2,4-D 6 mg/L, casein hydrolysate (CH, 200 mg/mL stock solution) 300 mg/L, sucrose 30 g/L, agar 7 g/L and pH 5.8. After the growth of the callus, *C. dactylon* cell suspension cultures were cultivated on 100 mL autoclaved flask with relevant nutrient medium (25 mL/flask), the compounds were 1/2 MS medium (A 0.625 mL, B 62.5 μL, C 125 μL and D 62.5 μL), 2,4-D (12.5 μL), sucrose (0.75 g), CH (25 μL, added after autoclaving) and pH 5.6. The *C. dactylon* cell suspensions were cultured under the same condition as *L. minor*.

Both *C. dactylon* and *L. minor* cell suspension cultures were sub-cultured every 14 days by taking 1 g fresh weight of the cells into fresh medium via a spoon-shaped sieve (0.8 mm mesh width). Before application, the sub-cultured cells (1 g/flask) were again cultured with fresh medium for 10 and 18 days (according to the results of time course tests on the cell growth rates, date are not published), respectively. During application, ^{14}C-propanil (10 μg, 25 kBq) was injected into each flask, and then the cell suspensions were incubated in darkness for 48 hr (*C. dactylon*), 72 hr (*C. dactylon*) and 7 days (*L. minor*) at 27°C with shaking (110 r/min), respectively. All experiments were performed in triplicates.

1.3. Extraction

After incubation, plant fresh weights were measured firstly and then the plants were completely crushed by an Ultra turrax and extracted with the Bligh–Dyer solution (chloroform/methanol, 2/1, V/V) (Bligh and Dyer, 1959). Plant debris and soluble fractions (plant extract) were separated by means of suction filtration (0.45 μm). A modified Bligh–Dyer solution (chloroform/methanol/H_2O, 2/1/0.8, V/V/V) was used to wash the debris during filtration. The nutrient medium of the plant and control systems were extracted by ethyl acetate (3 × 100 mL × 30 min). The extraction process of aseptic plants was similar to that of the intact plants. The filter papers with adhering plant debris were air-dried and subjected to combustion analysis ($^{14}CO_2$) by a Biological Oxidizer (OX 500, Zinsser/Harvey Instruments, Frankfurt, Germany).

Similarly, the fresh weights of cell suspensions were determined after separation from medium (suction filtration: 0.45 μm), then the cells were soaked in Bligh–Dyer solution (30 mL) and frozen at −18°C for 12 hr. The cell structures were destroyed by ultrasonification for 10 min (Bandlin Sonopuls HD 200, Berlin, Germany), following with re-filtration (0.45 μm) and washing by the modify Bligh–Dyer solution. The nutrient media of the cell cultures were extracted by ethyl acetate (2 × 20 mL × 10 min). The cell debris and the filter paper were air-dried and combusted in Biological Oxidizer.

1.4. Analysis

Duplicate samples (1 mL) were taken from plant extracts, cell extracts and medium extracts in order to detect the radioactivity (Liquid Scintillation Counter: Beckman LS 6500, Krefeld, Germany). The extracts were concentrated by rotary evaporation (EL 130, Büchi, Flawil, Swizerland) and dissolved in 10 mL methanol for radio-TLC (Thin Layer Chromatography) analysis: The samples (5000–10,000 dpm/100–500 μL) were placed on silica pre-coated TLC-plates (SIL G-25 UV 254, 0.25 mm, Macherey–Nagel, Düren, Germany) and developed in the solvents mixture A (dichloromethane/toluene of 9:1, V/V) and B (dichloromethane/acetone of 10:1, V/V). After developed in the TLC solvents A and B, R_f values of the non-labeled standards were propanil R_f: 0.49–0.55, 3,4-dichloroaniline (DCA) R_f: 0.65–0.72, 3,4-dichloroacetanilide (DCAA) R_f: 0.33–0.40, and N-(3,4-dichlorophenyl)-β-D-glucopyranosylamine (Glu-DCA) R_f: 0.00–0.04. Metabolites of propanil and parent propanil were identified by means of co-chromatography (the same R_f value) with the reference compounds 3,4-dichloroaniline (DCA), 3,4-dichloroacetanilide (DCAA) and N-(3,4-dichlorophenyl)-β-D-glucopyranosylamine (Glu-DCA).

Then the residual samples were further concentrated to 1 mL and filtrated (0.45 μm, Macherey–Nagel) before HPLC measurement. The HPLC system (HP Agilent 1200, Bad Homburg, Germany) contained a radio detector (raytest Ramona Star Serial, Straubenhardt, Germany) and a diode array detector (DAD) (250 nm, 350 nm; HP, Bad Homburg, Germany). Separation was performed on a Nucleosil C18 column (250 × 4 mm, cs-chromatographie, Langerwehe, Germany) and a corresponding pre-column (25 × 4 mm) under a flow of 1 mL/min with solvents A (H_2O + 0.1% acetic acid) and B (methanol/acetonitrile of 4/5 (V/V) + 0.1% acetic acid): A/B 80:20 (V/V) for 5 min, linear 5 min gradient to 45% B, isocratic B (45%) for 15 min and then again linear 5 min gradient to 85% B, isocratic B (85%) for 5 min and return to initial conditions in 10 min and isocratic for 5 min. Samples (20–100 μL) containing 50,000 dpm radioactivity were injected via an autosampler into the HPLC system and were first measured by DAD, and then the corresponding fractions were mixed with scintillation cocktail (Zinsser Analytic quick safe Flow2, Great Britain) for detection by a radio detector. Prior to the samples, the reference compounds were similarly examined. The retention times (Rt) of the non-labeled references using an optimized HPLC program were: propanil (28.75 min), DCA (21.83 min), DCAA (24.38 min) and Glu-DCA (14.15 min).

After HPLC analysis, prominent signals (indicating high radioactivity) were collected and again injected into HPLC for fragmentation (without pumping cocktail). The eluted fractions of different peak areas were collected and prepared for GC–EIMS (gas-chromatography coupled to a mass-spectrometer with electron impact ionization) (GC: Agilent 6890 N; MSD: Agilent 5973, Waldbronn, Germany). The GC–EIMS contained an Optima-35-MS capillary column (30 m × 0.25 mm, 0.25 μm film

thickness; Macherey–Nagel, Düren, Germany). Carrier gas was Helium (1.0 mL/min), injection volume 1 μL (manual injection). The temperature program was: injector temperature 250°C, oven temperature 50°C ramped at 10°C/min to 280°C, followed by another ramp to 240°C and finally to 270°C. MS temperatures were 230°C (source) and 150°C (quadrupole). MSD (mass selective detector) operated in full scan electron (m/z 50–550) with 70 eV ionization energy and the fractions were identified by comparing the MS spectra with standard substances (propanil, DCA and DCAA) and corresponding products reported in the NIST library.

2. Results

2.1. Hydroponic plant systems

The distributions of ^{14}C-residues in hydroponic plant systems are shown in Table 1. In the systems with plant *C. dactylon*, portions of ^{14}C found in plant extracts were 4.73% of the applied radioactivity (AR), whereas the corresponding non-extractable residues (NER) amounted to 7.73%. Thus after incubation (7 days), only 12.46% of AR was taken up by these plants, while the majority remained in the nutrient medium (73.36% of AR). Portions of root NER were nearly the same as those of the root and the shoot extracts, whereas shoot NER were twice that of the residues in the shoot extract. Radioactivity appeared in stem as well as leaf and its portion was higher than that in the root, which indicates noticeable acropetal transportation of ^{14}C in these plants. Contrasting with *C. dactylon*, higher uptake was observed in *N. nucifera* and *B. pilosa*. In these systems, only 22.35% and 9.41% of AR remained in the medium. The majority of ^{14}C found in both plants was present as non-extractable residues (NER). In *N. nucifera*, the NER fractions were 38.52% (root/shoot: 5), whereas extractable fractions amounted to 13.84% (root/shoot: 4.5) of AR. Similarly, higher portions of radioactivity were detected in the NER fraction derived from plant *B. pilosa* (47.94% of AR, root/shoot: 45.1). Correspondingly, extractable fractions were 28.61% (root/shoot: 24.8). With all plant species studied, ratios of NER and extractable fractions were considerably above 1 (*C. dactylon*: 1.6; *N. nucifera*: 2.8; *B. pilosa*: 1.8). Additionally, ^{14}C pesticide residues in the shoots of *N. nucifera* and *B. pilosa* were lower than that of the corresponding roots indicating lower translocation of pesticide residues from root to shoot, as compared to *C. dactylon*. The ^{14}C pesticide residues

found in the nutrient media of the controls (without plants) were 99.59% (*C. dactylon* medium), 84.34% (*N. nucifera* medium) and 90.04% (*B. pilosa* medium). Mineralization in all hydroponic plant systems was less than 0.05% of AR, i.e., slightly higher than that of the controls, except plant *B. pilosa*. In the present investigation, recoveries of ^{14}C ranged from 74.71% to 99.57%. Propanil equivalents found in the intact plants were 94.17 mg/kg (plant fresh weight) in plant *N. nucifera*, 51.03 mg/kg in plant *B. pilosa* and 63.37 mg/kg in plant *C. dactylon*.

Residues in the extracts were first characterized by radio-TLC (Fig. 2a). In the system of *B. pilosa*, propanil in plant and medium amounted to 12.87% and 6.11% of AR, respectively. Although DCA was not found in the plant extract, it is still considered as a metabolite since its conjugate, Glu-DCA, was detected in the plant extractable fractions (9.69% of AR). With respect to the medium in the control, propanil only amounted to 7.22%. DCA (23.18%) and Glu-DCA (18.83%) were the major portions. In the assays with *N. nucifera*, propanil was rapidly absorbed by the plant. After 7-day incubation, 1.28% of AR in the plant extractable fraction was identified as propanil. The analysis showed that metabolic products DCA and Glu-DCA were also present in plant extract. The medium contained propanil (2.88%), DCA (5.22%) and Glu-DCA (8.90%). The control (system without plants) contained propanil (5.5%), DCA (40.58%) and Glu-DCA (14.55%). Only small amount of radioactivity was absorbed by *C. dactylon*: the identified parent compound and its metabolic products (DCA and Glu-DCA) ranged from 0.34% to 1.71% of AR. The radioactivity in the medium extract was identified as propanil (24.03%) and DCA (26.5%). However, in the medium extract of the control, the portion of propanil amounted to 93.06% of AR, indicating that only a minor portion of propanil was metabolized by *C. dactylon* rhizosphere microorganisms. This finding contrasts with those of plants *B. pilosa* and *N. nucifera*.

Samples were then prepared for radio-HPLC analysis and the results are shown in Fig. 3a. Propanil and its metabolites (DCA and Glu-DCA) were detected in all fractions of *B. pilosa* and *N. nucifera* (except the medium of control from *N. nucifera*). In *B. pilosa*, propanil was the major fraction present in plant and medium extracts amounting to 13.42% and 6.10% of AR, respectively. However, in the control, DCA was identified as the main component (44.68%) besides propanil (42.71%). In contrast to *B. pilosa*, propanil was only a minor component in the soluble fractions of plant *N. nucifera*, i.e., plant and medium extracts, ranging from 0.7% to 0.9% of AR. Metabolites

Table 1 – Distribution of radioactivity in the hydroponic plant systems after 7 days of incubation with 20 mg/L (*C. dactylon*)/ 10 mg/L (*B. pilosa* and *N. nucifera*) and 42 kBq propanil.

Hydroponic culture	Plant extract	Plant NER	NM	^{14}CO$_2$	Recovery
	Percentage of applied radioactivity (%)				
C. dactylon	4.73 ± 1.06	7.73 ± 1.45	73.36 ± 5.68	0.03 ± 0.00	85.85 ± 4.12
	–	–	99.57 ± 3.33 [a]	0.02 ± 0.01 [a]	99.59 ± 3.34 [a]
N. nucifera	13.84 ± 1.52	38.52 ± 4.94	22.35 ± 1.48	0.05 ± 0.01	74.76 ± 6.58
	–	–	84.32 ± 1.02 [a]	0.02 ± 0.00 [a]	84.34 ± 1.02 [a]
B. pilosa	28.61 ± 0.93	47.94 ± 5.54	9.41 ± 4.89	0.01 ± 0.00	85.97 ± 1.38
	–	–	90.02 ± 2.29 [a]	0.02 ± 0.01 [a]	90.04 ± 2.29 [a]

NM: nutrient medium; –: no data.
[a] Control: All values are mean ± SD (n = 3).

Fig. 2 – Results of radio-TLC analysis of the soluble fractions obtained from (a) hydroponic plant systems (*B. pilosa*, *N. nucifera* and *C. dactylon*), (b) cell suspension cultures (*C. dactylon* and *L. minor*) and (c) sterilized hydroponic plants (*L. minor* and *L. gibba*). TLC: Thin Layer Chromatography.
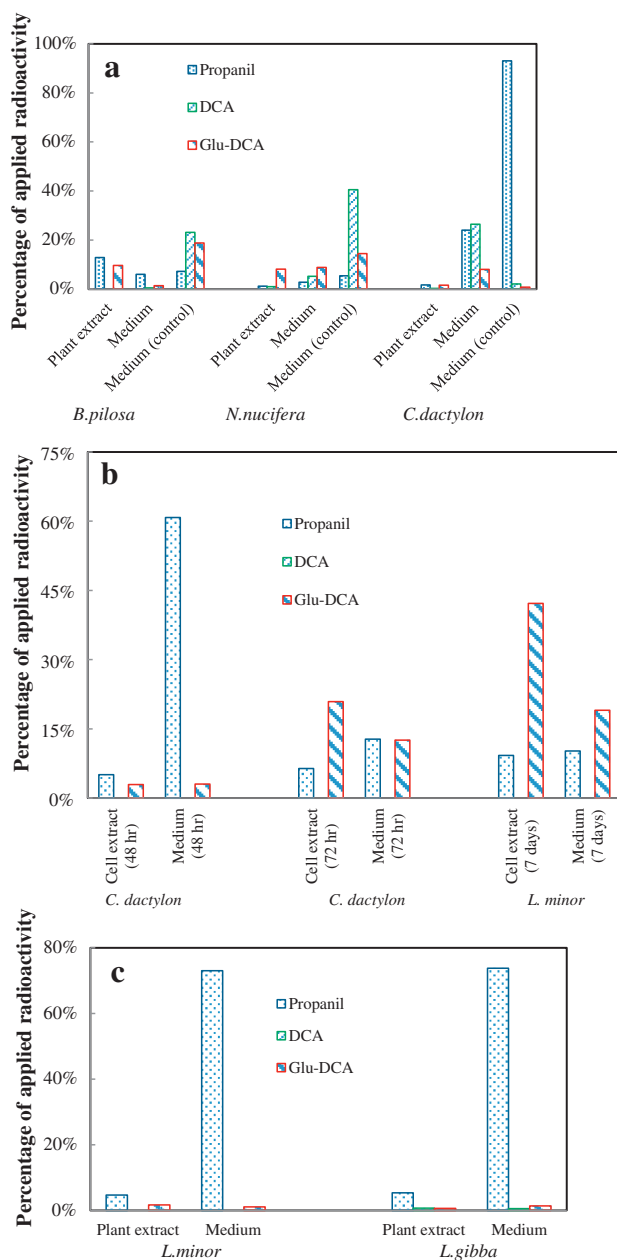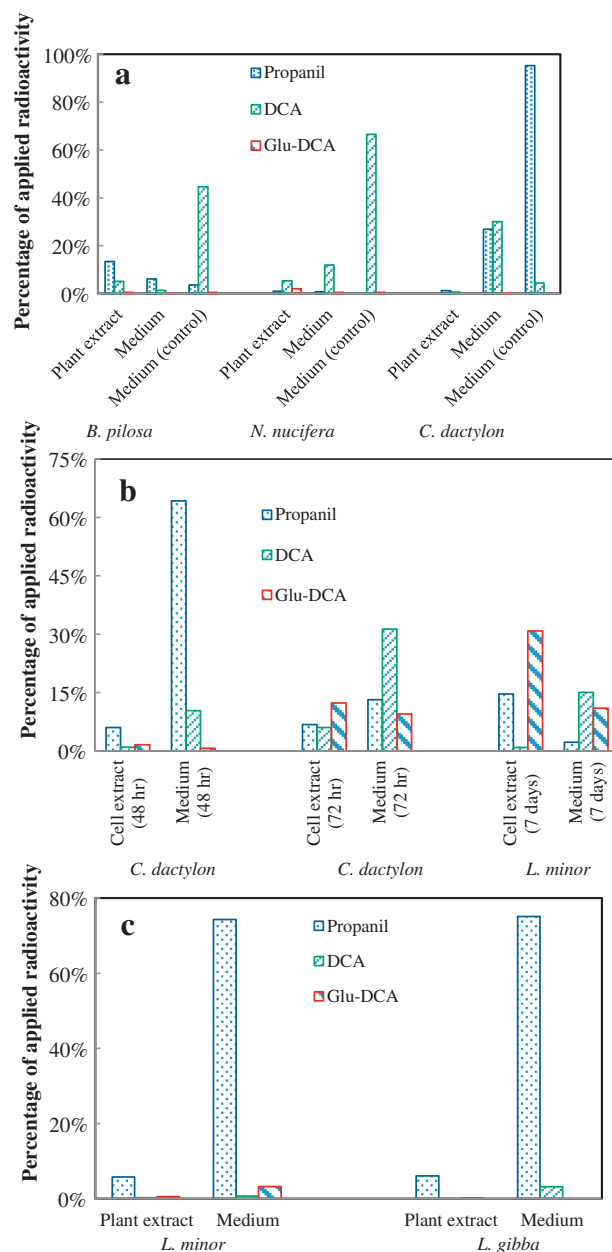

Fig. 3 – Results of radio-HPLC analysis of the soluble fractions obtained from (a) hydroponic plant systems (*B. pilosa*, *N. nucifera* and *C. dactylon*), (b) cell suspension cultures (*C. dactylon* and *L. minor*) and (c) sterilized hydroponic plants (*L. minor* and *L. gibba*).

identified were DCA and Glu-DCA; these were present in the plant extract (5.35% and 1.94%) and the nutrient medium (0.52% and 11.94%). In the control, DCA was the major portion (66.57%). Since no propanil was detected it appeared the herbicide was metabolized by rhizosphere microbes of *N. nucifera* in a noticeable short period (7 days). Thus, the radioactivity absorbed by *N. nucifera* consisted of propanil and its metabolites, especially DCA. Concerning *C. dactylon*, portions of propanil in plant extract were above 1.15% of AR, DCA amounted to 0.66%. The main

portions of the radioactivity remaining in the medium were identified as propanil (26.79%) and DCA (29.99%).

Portions of propanil and DCA identified by HPLC from the soluble fractions of hydroponic plant systems were similar as those pre-analyzed by TLC, except the media of the controls of *B. pilosa* and *N. nucifera*. In these samples, lower portions of propanil were detected by HPLC. In some cases, concentrations of DCA and DCA-conjugates (Glu-DCA) determined by HPLC analysis differed only slightly from those by TLC analysis. DCAA was not detected in the test systems.

2.2. Plant cell cultures and aseptic plants

Portions of ^{14}C found in cell suspensions increased with time (Table 2). About 10.59% (48 hr) and 39.22% (72 hr) of AR were absorbed by *C. dactylon* cells. Uptake of ^{14}C by *L. minor* cells amounted to 65.03% in 7 days. Major portions of radioactivity found in these cells were extractable, *i.e.*, 9.5% (48 hr), 33% (72 hr) and 53.8% (7 days) of the AR, respectively. Only minor portions of the radioactivity were bound to insoluble cell components, *e.g.*, polymers of the cell wall, thus forming the insoluble fractions amounting to 1.1%, 6.2% and 11.2%, respectively. As compared to the corresponding hydroponic plant systems, the ratios of insoluble to soluble fractions in the experiments using cell suspensions were particularly low ranging from 0.11 to 0.21. Remaining ^{14}C residues were detected in the media; these were 75.66% (48 hr), 53.93% (72 hr) and 35.55% (7 days), respectively. In case of axenic plants (*L. minor* and *L. gibba*), only minor portions of ^{14}C were taken up by the plants after 12 days of incubation (*L. minor*: 7.92%; *L. gibba*: 8.35%). As with the cell suspension cultures examined, extractable fractions in the plant tissue ranged from 6.76% (*L. minor*) to 6.99% (*L. gibba*), but NER concentrations were low (1% of AR) in both species. These results are in line with findings of Fujisawa et al. (2006). Ratios of the amounts of NER and extractable residues in these two aseptic plants were 0.17 and 0.2, respectively. The radioactivity remaining in the nutrient medium of the axenic plants was about 78% of AR for both species. Total recoveries of the radioactivity were 86% for both *Lemna* species. Regarding the axenic plants, propanil equivalents were 0.74 mg/kg (*L. minor*) and 0.51 mg/kg (*L. gibba*). In the cell suspensions, absorbed amounts were 0.96 mg/kg propanil equivalents (*C. dactylon* 48 hr), 1.71 mg/kg (*C. dactylon* 72 hr) and 2.03 mg/kg (*L. minor* 7 days).

Radio-TLC analysis (Fig. 2b) indicated that the metabolites identified in the *C. dactylon* cell suspension cultures were the same as those in the intact plant itself. After 48 hr incubation, the ^{14}C-peaks detected in the cell extracts were identified as propanil (5.09%) and Glu-DCA (2.92%). Similarly, propanil and Glu-DCA were also found in the medium; portions amounted to 60.84% and 3.04%, respectively. These substances were also identified in the cell and medium samples obtained after an incubation of 72 hr. In *L. minor* cell suspensions (7 days of incubation), percentages of propanil amounted to 9.24% in the cells. Glu-DCA was a major metabolite in the cells (42.22%) and medium (19.06%). In the aseptic plants (Fig. 2c), propanil

was confirmed to be the major portion in both plants and medium, which indicated that the degradation of propanil under such conditions was especially low. The metabolite portions ranged between 0.6% and 1.7% of AR in both plants and medium extract.

The corresponding HPLC results (Fig. 3b) indicated that propanil and DCA were found in the *C. dactylon* cells after 48 hr of incubation amounting to 6.03% and 1% of AR, respectively, whereas about 10-fold higher portions were found in the medium. After 72 hr, the propanil content of the cells was similar to that observed after 48 hr, whereas portion of DCA increased from 1% to 6% of AR. Simultaneously, the propanil portion in the medium had decreased to 13.13% and the DCA portion had increased to 31.33% of AR. Contrasting with the data resulting from the intact plant of *C. dactylon*, another metabolic product, Glu-DCA, was found in the cells and the corresponding media with increasing amounts during incubation. In the cell extract of *L. minor*, propanil amounted to 14.62% of AR, DCA to only 0.91%, and the majority portion was Glu-DCA (30.83%). The amount of propanil in the medium extract was 2.22% and those of DCA and Glu-DCA were 15.06% and 10.96%, respectively. Additionally, a polar fraction (not present in the figure) with Rt = 11.73 min appeared in *L. minor* cell extract and medium amounting to 0.96% and 2.5%, respectively. This polar fraction, however, was neither found in the aseptic *L. minor* plant extract nor in the corresponding media. In the soluble fractions of the aseptic plants, propanil amounted to 5.71% (*L. minor*) and 6.06% (*L. gibba*). The metabolic products in both axenic plants amounted to only 0.01%–0.19% (DCA) and 0.14%–0.52% (Glu-DCA).

Concerning the aseptic plants and cell suspensions, portions of propanil identified by TLC were also in line with that obtained from HPLC. The main difference concerned DCA and Glu-DCA concentrations, which were similar to those of hydroponic plant systems. The sum of DCA and Glu-DCA in each soluble fraction identified by HPLC was equal to the relevant Glu-DCA portions detected by TLC. A reasonable explanation could be that during the separation on TLC plates DCA was liberated from Glu-DCA, as Glu-DCA was proven to be hydrolytically labile (Winkler and Sandermann, 1992).

Based on the signals of radio-HPLC, selected extracts from hydroponic cultures and cell suspensions were subjected to preparative HPLC. Afterwards, these fractions were isolated, concentrated and prepared for gas-chromatography coupled to a mass-spectrometer with electron impact ionization (GC–EIMS). The identities of propanil and DCA could be confirmed but not

Table 2 – Distribution of ^{14}C in axenic cell suspension cultures (48 and 72 hr: *C. dactylon*; 7 days: *L. minor*) and axenic plants (incubated for 12 days) with 10 μg (25 kBq; cell suspension cultures) and 0.05 mg/L (32 kBq; axenic plants) of propanil.

Axenic plants/cell suspensions		Plant/cell extract	NM	Plant/cell NER	Recovery
		Percentage of applied radioactivity (%)			
Cell suspension cultures	*C. dactylon* (48 hr)	9.51 ± 2.11	75.66 ± 9.34	1.08 ± 0.17	86.25 ± 8.1
	C. dactylon (72 hr)	33.03 ± 7.85	53.93 ± 5.53	6.19 ± 2.44	93.15 ± 5.95
	L. minor (7 days)	53.82 ± 1.97	35.33 ± 0.59	11.21 ± 0.99	100.37 ± 1.19
Axenic plants	*L. minor* (12 days)	6.76 ± 0.47	78.13 ± 3.02	1.16 ± 0.07	86.06 ± 2.59
	L. gibba (12 days)	6.99 ± 0.73	78.21 ± 1.02	1.36 ± 0.17	86.56 ± 0.37

All values are mean ± SD (*n* = 3).
NER: non-extractable residues.

that of the polar and non-volatile Glu-DCA metabolite. Two fractions remaining non-identified after GC–EIMS were re-analyzed by TLC using successively two solvents with increasing polarity: solvent 1, methylene chloride; solvent 2, chloroform/methanol/H$_2$O of 65/25/4 (V/V/V). The fraction with HPLC Rt of 12–16 min revealed a single peak with R_f = 0.35 (after solvent 2), which corresponded to that of the reference substance Glu-DCA (Bockers et al., 1994). The HPLC fraction with Rt of 10–12 min was immobile on the TLC plate even with solvents 1 and 2 successively. This portion of radioactivity may consist of a malonylated derivative of Glu-DCA, 6′-O-malonyl-N-(β-D-glucopyranosyl)-3,4-dichloroaniline (Mal-Glu-DCA), which has previously been reported by Bockers et al. (1994) in a metabolism study of DCA in soybeans and wheat (Bockers et al., 1994).

In summary, the metabolism of propanil in the tested species led to the formation of the primary metabolite DCA and to the secondary product Glu-DCA. Mal-Glu-DCA was only detected in the *L. minor* cell suspension culture, which was just confirmed by analogy. Mal-Glu-DCA may have also been present in other examined species, but may have been hydrolyzed during work-up of the samples.

3. Discussion

3.1. Extractable fractions and non-extractable fractions (NER)

We investigated the biotransformation of the ^{14}C-ring-labeled herbicide propanil in plants, dominating in the littoral zone of the TGR, and incubated the compound in non-aseptic hydroponic plant systems, aseptic plants and plant cell suspension cultures. All tested plants incorporated the parent compound and formed metabolites, *i.e.*, DCA, Glu-DCA, which have also been detected in other plant and plant cell culture studies (Bockers et al., 1994; Gareis et al., 1992; Ray and Still, 1975; Schmidt et al., 1994). The metabolite Mal-DCA may have been produced (but was not detected) since malonyl acid as a natural plant constituent and the associated N-malonlyltransferases are able to form corresponding conjugates with xenobiotics, such as chlorinated anilines (Sandermann et al., 1991). Some conjugated xenobiotics like the O-(malony) glucoside of pentachlorophenol and Mal-DCA are released from the cells and stored in the apoplast (Winkler and Sandermann, 1989). Conjugates with reasonable lipid solubilities may then diffuse across the lipophilic membranes of the cells to reach the nutrient medium (Camper and McDonald, 1989). Correspondingly, in *C. dactylon* cell suspensions, the amounts of metabolic products in the medium were higher than in the cells. However, the ratio of the amounts of ^{14}C-metabolites between cells and medium varied between the plants and depended on the duration of incubation. If sugar-conjugates were formed they are prone to hydrolytic cleavage afterwards, as reported for the hydrolysis of Glu-DCA and the conversion into the N-malonyl conjugate (Schmidt et al., 1994; Winkler and Sandermann, 1992). DCAA was found in propanil treated rice plant and *L. minor*, the formation being catalyzed by acetyltransferase (Mitsou et al., 2006). This metabolite has neither been found in *L. gibba* (Fujisawa et al., 2006) nor in our study. Unlike in microorganisms, oxidative enzymes in the plants are obviously unable to dimerize DCA, thus the

relevant products such as TCAB and other azo-products are not generated from plants.

In the control of *B. pilosa* (medium without plant), metabolites were the major identified ^{14}C-substances. This indicates that propanil was more rapidly metabolized by rhizosphere microorganisms of *B. pilosa* in contrast to the plant itself. Similar results were obtained in *N. nucifera*. In addition, the propanil portion in the medium extract of *B. pilosa* was higher than that of the control, which seem to indicate that the biodegradation of propanil in the rhizosphere was inhibited, *e.g.*, by root exudation of interfering natural metabolites. Although lower amounts of applied radioactivity were taken up by *C. dactylon*, absorbed propanil equivalents were similar as in the other two plants due to the higher concentration and the smaller biomass of the plant. All propanil equivalents in the experiments exceeded previous proposed trigger values (0.1–0.5 mg/kg, dry commodities) of the herbicides, similar to the findings summarized by Sandermann (2004).

High amounts of NER were formed in the hydroponic plant systems in which chemicals may rapidly be taken up from the water phase, whereas under field conditions, long-term exposure can result in high amounts of pesticide non-extractable residues in plant tissues. Concerning aseptic plants, formation of NER was lower than that in the cell suspensions. The uptake of propanil by *L. minor* in our study differed from that of a previous study (Mitsou et al., 2006), in which intact plants (non-aseptic) and the adhering microorganisms were grown in a sterilized nutrient. The findings emphasized that plant uptake and formation of NER may arise by mutual action between plants and microorganisms. The ratio of NER and extractable residues in the intact plants was higher than that in the cell suspensions, indicating that the pesticide residues can not only be bound to the cell wall surrounding every cell but also for instance to xylem, cutin and phloem structures (Corio-Costet et al., 1991; Feely and Crouch, 1997). Besides, floating plants such as duckweeds contain low amounts of lignin; thus, less NER are formed, in contrast to other vascular plants. Also, in contrast to cell cultures, radioactivity in the corresponding axenic plants was lower, which was due to the higher cellular uptake of the pesticide by the cells of the suspension culture. We conclude that *L. minor* is susceptible towards the herbicide propanil. Pesticide equivalents (both extractable and non-extractable fractions) taken up in the examined plants exceeded the maximum residue levels (MRLs) of 0.01 mg/kg that are considered protective for consumers according to European guidelines (EFSA, 2013). Although non-extractable residues are immobile and thought to be less or even non-bioavailable, such residues in plants are not without environmental concern, since NER may be partly digested by consumption by human or livestock and even trace amounts of toxic pollutants may induce adverse effects in exposed organisms (Akhtar et al., 1992). Some pesticide NER in plants have been reported to be highly bioavailable, *i.e.*, [ring-U-^{14}C] chloroanilines/lignin could release up to 66% of the initial NER in the form of simple chloroanilines after feeding to rats (Sandermann et al., 1990). In the field, plants contribute most portions of the organic input into wetlands and are the main source of forming organic matter both in soil and sediments. It has to be taken into account, that also xenobiotic residues in plants may be released after plant decay simultaneously leading to freely dissolved compounds that might either

be rapidly degraded by microorganisms or in case of persistence may lead to toxic effects. A corresponding study indicated that the release of propanil-incubated plant NER is quite low (unpublished).

Mineralization of ^{14}C-ring-labeled propanil was higher in a plant–soil system in contrast to that in soil only (Sutherland, 1976), probably due to the fact that plant roots can transport oxygen to the rhizosphere area, causing an increase of enzymatic activity of rhizospheric microbes; thus, the rate of biodegradation of xenobiotics may increase (Wetzel, 1993). Our results are in line with this previous study, but the mineralization rates were particularly low (<0.05% of AR), as compared to those obtained from studies on the fate of propanil and DCA in soils and sediments (Hennig, 2013; Zhang, 2013).

3.2. Bioavailability

Propanil is a phytotoxic substance and treated crops are consumed by humans and animals. Therefore, the toxicity and bioavailability of both parent compound and metabolites need to be investigated. As described above, propanil equivalents in all plant systems tested exceeded the proposed trigger values of European authorities (EFSA, 2013), although the particularly high amount of pesticide residues was mainly due to the optimized uptake conditions, i.e., hydroponic culture systems. *N. nucifera*, an aquatic plant, is used for food around the world, especially in Asia, and thus analysis of the residual levels of pesticides in the plants is necessary. The acute toxicity of propanil for animals and humans is low, and it is considered neither genotoxic, carcinogenic nor mutagenic (EFSA, 2011; EPA, 2003). The MRL of 0.01 mg/kg propanil in plant tissue is protective for consumers according to European guidelines (EFSA, 2013), which is lower than that in other countries, like China (MRL 2 mg/kg; http://www.chinapesticide.gov.cn). Floating plants, *e.g.*, duckweeds represent a food source for aquatic herbivores and other organisms.

Xenobiotics such as propanil ($\log K_{ow}$ *ca.* 3) with low water solubility and a high bio-concentration potential are expected to be accumulated in organisms via food chain. Propanil is extensively metabolized to DCA, which is also a metabolite of other pesticides like linuron and diuron (EFSA, 2013). Attention must be given to DCA because of its persistence rendering the environmental concentration often higher than that of the parent compound (Gooddy et al., 2002; Still and Mansager, 1969). Regarding DCA-conjugates such as sugar or malonyl-conjugates, less information on toxicity is available, but in general, these conjugates are rapidly cleaved in organisms enzymatically. Additionally, these conjugates are plant degradation products during three-phased processes (Phase I: transformation, Phase II: conjugation, Phase III: compartmentation), thus they are less toxic to plant and other organisms (Sandermann, 2004; Schmidt, 1999). Only limited data of the secondary metabolites TCAB and TCOAB formed by condensation of DCA exist (Poland et al., 1976; Witt et al., 2000). Those compounds were found at low concentrations in fields treated with pesticides releasing DCA. Since the azo functional group of the DCA dimers implies some mutagenicity potential, corresponding ecotoxicity analyses are currently performed in our laboratory.

4. Conclusions

Plants dominating in the TGR and adapted to the specific hydrological conditions in association with their rhizospheric microorganisms are able to rapidly take up and degrade propanil, producing DCA which is then further conjugated with sugar compounds, *i.e.*, Glu-DCA. As high amounts of non-extractable residues were formed, reducing the bioavailability of both propanil and its metabolites, we therefore conclude that the vegetation in the littoral zone of the TGR, both natural and artificial, can accumulate pollutants in the TGR which are released by agricultural practice and from re-suspended contaminated sediment. The riparian vegetation acts as an ecological barrier and according to our findings plays a significant role in the fate of environmental pollutants, such as the herbicide propanil.

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