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Uptake and metabolism of human pharmaceuticals in plants

Identification of metabolites and specification of the defense enzyme systems under pharmaceutical exposure

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List of abbreviations

APOX	Ascorbate peroxidase
°C	degree Celsius
CAT	Catalase
COX	cyclooxygenase
C_T	threshold cycle
CYP	Cytochrome P450
cDNA	complementary deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EC_{50}	half maximal effective concentration
ε	extinction coefficient [$mM^{-1}cm^{-1}$]
FW	fresh weight
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
g/L	gram per liter
GR	Glutathione reductase
GST	Glutathione-S transferase
GT	Glycosyltransferase
h	hour
HPLC	High-performance liquid chromatography
kDa	kilodalton
L	liter
LC-MS	Liquid chromatography-mass spectrometry
mg/L	milligrams per liter
µg/L	micrograms per liter
mL	milliliter
µL	microliter
mM	millimole
µM	micromole
MS/MS	Tandem mass spectrometry
NSAIDs	non-steroidal anti-inflammatory drugs
nm	nanometer
OD	optical density

PCPs	personal care products
PCR	polymerase chain reaction
POX	Peroxidase
Q-PCR	quantitative- or real time polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RT	Reverse transkriptase
v/v	volume to volume ratio
WWTPs	wastewater treatment plants

List of abbreviated chemicals

4-NPG	4-Nitrophenyl ß-D-glucuronide
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
$Ca(NO_3)_2 \cdot 4H_2O$	Calcium nitrate tetrahydrate
CDNB	1-chloro-2,4-dinitrobenzene
$CuSO_4 \cdot 5H_2O$	Copper(II) sulfate pentahydrate
DCNB	1,2-dichloro-4-nitrobenzene
DTE	Dithioerythritol
EDTA	Ethylenediaminetetraacetic acid
FeNa-EDTA	Iron(III)-sodium ethylenediaminetetraacetate hydrate
GSH	Glutathione reduced
GSSG	Glutathione oxidized
H_3BO_2	Boric acid
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
$MnCl_2 \cdot 4H_2O$	Manganese(II) chloride tetrahydrate
$MnSO_4$	Manganese(II) sulfate
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
NADPH	Nicotinamide adenine dinucleotide phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
NH_4NO_3	Ammonium nitrate
PMSF	Phenylmethanesulfonyl fluoride
pNBC	p-nitrobenzylchlorid
pNBoC	p-nitrobenzoylchlorid
pNPA	p-nithrophenylacetat

TCP	Trichlorophenol
TFA	Trifluoroacetic acid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
UDPG	Uridine 5´-diphosphoglucose disodium salt
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate

Abstract

The fate of pharmaceuticals in our environment is a current problem and a very important issue for environmental and health research. We now use more synthetic drugs than ever before and allow these drugs to enter the environment, mostly in the municipal wastewater treatment plants. From there they can occur in effective concentrations and are often degraded slowly or incompletely. Thus they represent a major threat to the aquatic environment and to drinking water quality. The most common medications in wastewater are analgesics, lipid-lowering agents, anti-epileptics, antibiotics and beta-blockers, in decreasing order of the concentrations found.

In our research we have investigated the uptake and metabolism of paracetamol (N-acetyl 4-aminophenol) and diclofenac in Indian mustard (*Brassica juncea* L. Czern.) and Common Cattail (*Typha latifolia* L.). These drugs are two of the most widely used painkillers in Germany. The plants were grown in Hoagland liquid culture under controlled conditions (12 h light period, 16 °C and controlled humidity). After treatment with paracetamol (1 mM and 1 mg/L) and diclofenac (1 mg/L) samples were collected after 24, 72 and 168 hours. In case of *Typha* additional samples were taken after 30 days of incubation.

The characterization of paracetamol, diclofenac and their metabolites in the two plant species was carried out with LC-MS methods. Changes in enzyme activity of the anti-oxidative metabolism (APOX, GR and POX) were examined to characterize oxidative stress and defense mechanisms induced by the two pharmaceutical substances. Activity of Glycosyltransferase and Glutathione S-transferase was also investigated. These enzymes play a major role in the detoxification and degradation of xenobiotics in plants.

We demonstrated the uptake of paracetamol and diclofenac in both plants using LC-MS measurements; 24 hours after treatment both substances could be detected in root and leaf tissues.

The following paracetamol conjugates were identified: paracetamol-glutathione, paracetamol-cysteine and paracetamol-glucopyranoside. The degradation process is localized mainly in the roots, but we have found evidence of metabolism in leaves as well. Furthermore we were able to detect a significantly increased GST activity in the treated plants. These results suggest the involvement of different GST isoforms throughout the process of detoxification.

The following diclofenac conjugates were identified: 4'-hydroxy-diclofenac and OH-diclofenac glutathione. These two conjugates are also described in the metabolic route in mammalians. In addition we identified diclofenac glucopyranoside (conjugate with glucose), which is a plant-specific metabolite. These metabolites were found mainly in root tissues, suggesting metabolism is centered on the roots. In addition GST isoenzymes were found to play an important role in the degradation pathways as well. We were able to demonstrate their inducibility by diclofenac using RT-qPCR measurements.

Activity of antioxidative defense enzymes, especially POX, increased proportionally during exposure time. This indicates oxidative stress induced by exposure to the pharmaceuticals. APOX and GR show different reactions in *Typha* and *Brassica* plants. In *Typha* we detected increased APOX and GR activity after treatment with 1 mM and 1 mg/L for both drugs. In *Brassica* however, their activity was reduced. All enzymatic reactions portend a higher tolerance of *Typha* to the pharmaceuticals under investigation.

Zusammenfassung

Der Verbleib von Pharmazeutika in unserer Umwelt ist ein aktuelles Problem und ein sehr wichtiges Thema für die Umwelt- und Gesundheitsforschung. Wir nutzen heutzutage mehr synthetische Medikamente als je zuvor und diese Arzneimittel gelangen dann in die Umwelt, zumeist in die kommunalen Abwasserkläranlagen. Da sie dort in wirksamen Konzentrationen auftreten können und häufig nur langsam oder unvollständig abgebaut werden, bedeuten sie eine große Gefahr für die aquatische Umwelt und die Trinkwasserqualität. Die am häufigsten vorkommenden Medikamente im Abwasser sind Schmerzmittel, Lipidsenker, Antiepileptika, Antibiotika und ß-Blocker, in abnehmender Reihenfolge der gefundenen Konzentrationen.

Wir haben in unseren Experimenten Aufnahme und Stoffwechsel von Paracetamol (N-Acetyl 4-Aminophenol) und Diclofenac in Braunem Senf (*Brassica juncea* L. Czern.) und Rohrkolben (*Typha latifolia* L.) untersucht. Diese sind zwei der meistgenutzten Schmerzmittel in Deutschland. Die Pflanzen wurden in Hoagland-Flüssigkultur und unter kontrollierten Bedingungen (12 Stunden Lichtperiode, 16 ºC und kontrollierte Luftfeuchtigkeit) angezogen, mit Paracetamol (1 mM und 1 mg/L) und Diclofenac (1 mg/L) belastet und nach 24, 72 und 168 Stunden geerntet. Zusätzlich wurden *Typha* Pflanzen auch nach 30 Tage Inkubationszeit geerntet.

Die Charakterisierung von Paracetamol, Diclofenac und ihren Metaboliten in den Pflanzen wurde mit LC-MS Methoden durchgeführt. Die Änderungen von Enzymaktivitäten des antioxidativen Stoffwechsels (APOX, GR und POX) wurden untersucht, um den durch die Wirkstoffe ausgelösten oxidativen Stress und die Abwehrmechanismen in Pflanzen zu charakterisieren. Die Aktivitäten des Enzyms Glycosyltransferase und Glutathion S-Transferase, die im Entgiftung- und Abbauprozessen von Xenobiotika im Pflanzen eine wichtige Rolle spielen, wurden ebenso untersucht.

Mit HPLC-MS Messungen haben wir die Aufnahme von beiden Arzneimitteln in die Pflanzen nachgewiesen; nach 24 Stunden konnten wir sowohl Paracetamol als auch Diclofenac in den Wurzeln als auch in den Blättern detektieren.

Von Paracetamol wurde folgende Konjugate identifiziert: Paracetamol-Glutathion, Paracetamol-Cystein und Paracetamol-Glucopyranosid. Der Metabolismus findet zum größten Teil in den Wurzeln statt. Jedoch haben wir auch Hinweise auf Stoffwechsel in den Blättern gefunden. Zudem konnte in den Blättern der behandelten Pflanzen eine signifikante GST-Aktivitätssteigerung gegenüber verschiedenen Substraten gemessen

werden. Das Ergebnis deutet auf die Beteiligung der verschiedenen GST Isoformen in diesem Prozess hin.

Von Diclofenac wurden die folgenden Metaboliten identifiziert: 4‘-Hydroxy-Diclofenac und Hydroxy-Diclofenac-Glutathion, die auch im Stoffwechsel von Säugetieren beschrieben wurden, und Diclofenac Glucopyranosid (Konjugat mit Glucose), welches ein pflanzenspezifisches Stoffwechselprodukt darstellt. Die Metaboliten wurden zum größten Teil in Wurzeln gefunden, was auf einen wurzellokalisierten Stoffwechsel hindeutet. In diesen Abbauwegen spielen die GST Isoenzyme auch eine wichtige Rolle. Ihre Induzierbarkeit durch Diclofenac konnten wir mit RT-qPCR Messungen belegen.

Die antioxidativen Abwehrenzyme, vor allem Peroxidase, zeigen eine mit der Dauer der Behandlungszeit proportional ansteigende Aktivität. Dies deutet auf oxidativen Stress hin, der durch die Wirkstoffe hervorgerufen wurde. APOX und GR zeigen unterschiedliche Reaktionen in *Typha* und in *Brassica* Pflanzen. Während die Behandlung von *Typha* für beide Wirkstoffe eine erhöhte APOX und GR Aktivität zur Folge hatte, konnten wir eine Enzymhämmung in *Brassica* feststellen. Alle enzymatischen Reaktionen zeigen die bessere Toleranz der *Typha* Pflanzen auf die untersuchten Wirkstoffe.

1 Introduction

1.1 Pharmaceuticals in the environment

Since the industrial revolution the presence of anthropogenic compounds in the environment has been growing at an incredible pace; we are depending on the use of chemicals in everyday life more than ever. Besides "classical" organic pollutants like industrial chemicals, heavy metals, pesticides, fertilizers, detergents, surfactants, plasticizers and many others, a new group of impurities reached the focus of interest. Over the last decade and thanks to technical development in the field of environmental analysis we recognized the occurrence of pharmaceuticals and personal-care products (PCPs) like disinfectants, fragrances, and repellents in our environment (Halling-Sorensen et al. 1998; Ternes et al. 1998; Kolpin et al. 2002; Brausch and Rand 2011). Although these pollutants have been detected in low concentration until now (in the range of ng/L to μg/L), they may pose considerable environmental risk on people and ecosystems, because of the huge amount that enters the environment every day (Daughton and Ternes 1999). In the European Union about 3000 different active pharmaceutical ingredients are used in human and veterinary medicine. Every year hundreds of tons of the most frequently used drugs are being consumed in Germany alone (Table 1.1).

1.1.1 Entry and occurrence of pharmaceuticals in the environment

Pharmaceuticals are bioactive compounds designed to be stable to make them suitable for longer storage in the shelf or at the consumer's, stable against enzymes and acidic pH, to allow stomach passage, and to be water soluble for better resorption. These properties make such compounds at the same time mobile and persistent in the environment and may thus have ecotoxicological effects on non-target organisms.

In the European Union pharmacy makes up 25% of the total chemical production; 30,000 t of active substances per year are being produced in Germany and used in 50,000 different products for human medication. Another 3000 veterinary medicaments are in use containing 600 different active substances. Approximately 2,500 t of these compounds are being employed each year, and we have no data about pharmaceuticals used in animal fodder to increase yields and performance (Bayerisches Landesamt für Umwelt 2008). The most frequently utilized pharmaceuticals in human medicine are non-steroidal anti-inflammatory drugs (NSAIDs), beta blockers, blood lipid lowering agents, cancer therapeutics, neuroactive compounds, antibiotics and hormones.

Table 1.1 The most frequently used pharmaceuticals in Germany in year 2001. (Huschek und Krengel, 2003)

Compound	Consumed drugs in Germany 2001 (tons)
NSAIDs:	
Acetylsalicylic acid	836.26
Paracetamol	621.65
Ibuprofen	344.89
Diclofenac	85.8
Beta blockers:	
Metoprolol	92.97
Sotadol	26.6
Antiepileptic drugs:	
Carbamazepine	87.6
Antibiotics:	
Sulfamethoxacol	53.6
Doxycyclin	24.18
Ciprofloxacin	17.97
Hormones	
17α-Ethinylestradiol	0.05

Pharmaceuticals can enter the environment via different routes (Fig. 1.1). The main sources are the households and hospitals; after using in human medication the active compounds are both excreted in the urine and feces either intact or metabolized by enzymes to multiple metabolites and entered to the municipal wastewater system. In addition many drugs enter the wastewater directly as people flush them down the toilet instead of ensuring proper disposal. Another, related entry path develops from the use of crèmes and lotions containing pain killers in high concentrations. The largest part of the active ingredients (90%) remains on the skin and is washed off during body hygiene. Although wastewater treatment plants (WWTPs) are effective in cleaning the inlets from a wide range of contaminants, they are incapable to completely remove micro pollutants like pharmaceuticals and personal care products from the influent water. After the treatment process the outlet water in many cases contains at least marginal concentrations of active substances that reach surface water bodies (Lienert et al. 2007; Bartelt-Hunt et al. 2009)

(Table 1.2). Treated wastewater is often reused in agriculture as irrigation water. In many low-income countries even untreated wastewater is used for irrigation and leads to a direct exposure of soil to the pollutants (Kinney et al. 2006).

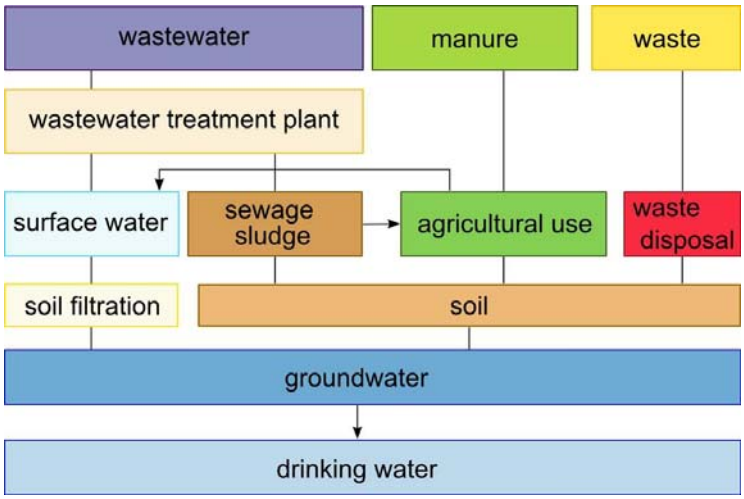


Figure 1.1 The main sources and fates of pharmaceuticals in the environment (adapted from Ternes, 1998).

From the soil, pharmaceuticals or their active metabolites can enter into the ground water and finally end up in our drinking water reserves (Heberer 1998; Rabiet et al. 2006). Pharmaceuticals can also enter the food chain via plant uptake from soil (Boxall et al. 2006; Redshaw et al. 2008). Furthermore by infiltrating surface waters the aquatic ecosystem is exposed to these pollutants (Cleuvers 2003; Lin et al. 2008; Daneshvar et al. 2010).

This problem for the ground water is even increasing as non-used drugs are frequently discarded into the regular household garbage and end up in waste disposal sites. Although in many cases the garbage is incinerated, leachates from municipal solid waste deposits can enter the soil and contaminate groundwater.

As mentioned above, yet another main source of pharmaceuticals is veterinary medicine and animal husbandry. Veterinary medicines mainly enter the terrestrial environment via manure and slurries. In addition some pharmaceuticals – mostly antibiotics used in fish farming – directly enter the aquatic environment.

Table 1.2 Concentrations of the most frequently used drugs in the aquatic environment.

Compound	Concentration (ng/L)	Aquatic environment	Reference
NSAIDs and painkiller agents			
Paracetamol	0.6	wastewater effluent	Ternes et al. (1999)
	1575	wastewater effluent	Kasprzyk-Hordern et al. (2009)
	662	surface water	Kasprzyk-Hordern et al. (2009)
Diclofenac	2,5	wastewater effluent	Heberer (2002)
	179	wastewater effluent	Kasprzyk-Hordern et al. (2009)
	49	surface water	Kasprzyk-Hordern et al. (2009)
	778	wastewater effluent	Daneshvar et al. (2010)
	72	surface water	Daneshvar et al. (2010)
Ibuprofen	28	surface water	Kasprzyk-Hordern et al. (2009)
	107	wastewater effluent	Daneshvar et al. (2010)
	31	surface water	Daneshvar et al. (2010)
Antiepileptic			
Carbamazepine	6300	wastewater effluent	Ternes (1998)
	1200	wastewater effluent	Huscheck (2001)
	826	surface water	Kasprzyk-Hordern et al. (2009)
	2997	surface water	Valcárcel et al. (2011)
Antibiotics			
Sulfamethoxazole	44	wastewater effluent	Kasprzyk-Hordern et al. (2009)
	4	surface water	Kasprzyk-Hordern et al. (2009)
	879	surface water	Valcárcel et al. (2011)
Ciprofloxacin	6	surface water	Valcárcel et al. (2011)
Lipid regulators			
Clofibric acid	730	wastewater effluent	Heberer (2002)
	250	wastewater effluent	Tauxe-Wuersch et al. (2005)
	8	surface water	Kasprzyk-Hordern et al. (2009)
	75	wastewater effluent	Kasprzyk-Hordern et al. (2009)
Bronchodilators			
Salbutamol	63	wastewater effluent	Kasprzyk-Hordern et al. (2009)
	3	surface water	Kasprzyk-Hordern et al. (2009)
	29	surface water	Valcárcel et al. (2011)
Beta blockers			
Metoprolol	6.3	wastewater effluent	Ternes (1998)
	1.18	wastewater effluent	Huscheck (2001)
	12	surface water	Kasprzyk-Hordern et al. (2009)

1.2 **Non-steroidal anti-inflammatory drugs and analgesic agents**

In the human body prostaglandins promote inflammation, pain and fever, support the blood clotting function of platelets and protect the lining of the stomach from the damaging effects of acid. Prostaglandins are produced by two cyclo-oxygenase (COX) enzymes, COX-1 and COX-2. Non-steroidal anti-inflammatory drugs block these enzymes and thereby reduce pain and inflammation (Peterson et al. 2010). However, common side effects of COX-1 inhibition are adverse stomach and intestinal reactions like irritation, bleeding or perforations. Still, many of them can be purchased over the counter.

NSAIDs can be poorly removed from municipal wastewater, and have been detected in significant concentration up to µg/L levels in wastewater effluents, surface waters, rivers and ground waters (Heberer 2002; Kasprzyk-Hordern et al. 2009; Valcárcel et al. 2011). The most frequently consumed painkillers in Germany are acetylsalicylic acid, paracetamol, diclofenac and, to a lower amount, ibuprofen. Worldwide diclofenac and ibuprofen can also be found among the most commonly detected NSAIDs in the environment.

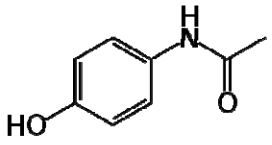
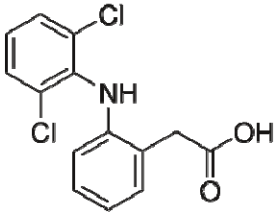
Structure	Data
	**Paracetamol** or acetaminophen Molecular mass: 151.17 g/mol IUPAC: N-(4-hydroxyphenyl)ethanamide N-(4-hydroxyphenyl)acetamide CAS number: 103-90-2
	**Diclofenac** Molecular mass: 296.147 g/mol IUPAC: 2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid CAS number: 15307-86-5

Figure 1.2 Chemical structure of the investigated pharmaceuticals paracetamol and diclofenac.

1.2.1 Paracetamol

Paracetamol (Figure 1.2) or acetaminophen (United States adopted name) has a unique position among analgesic drugs, since it is not clearly classified yet. Some sources categorize it as a non-steroidal anti-inflammatory drug; others do not assign it to this pharmaceutical group, but describe it as an aniline analgesic. The essential distinction between NSAIDs and paracetamol is the lack of anti-inflammatory and anti-rheumatic activity; the two systemic effects of paracetamol are analgesia and antipyresis (Bertolini et al. 2006).

The majority of paracetamol (approximately 90%) is metabolized in the liver; 40-67% of the drug is conjugated with glucuronide, 20-46% with sulphate and about 3% with cysteine forming inactive and harmless metabolites. On the contrary, 5-10% is oxidized by Cytochrome P450 enzymes, resulting in the formation of N-acetyl-p-benzoquinoneimine (NAPQI), a highly reactive and toxic intermediate compound. To avoid toxicity, NAPQI would be quickly conjugated by glutathione leading to cysteine and mercapturic acid metabolites. These conjugates and a small amount of uncharged paracetamol (approximately 2-5%) are finally excreted from the human body in urine (Bertolini et al. 2006).

1.2.2 Occurrence and fate of paracetamol in the environment

Paracetamol is frequently determined in wastewater influents in µg/L concentrations (Ternes et al. 1999; Kasprzyk-Hordern et al. 2009) and in spite of high removal efficiency it is often measured in ng/L concentration in surface waters (Wiegel et al. 2003; Radjenovic et al. 2007; Kasprzyk-Hordern et al. 2009).

In many papers paracetamol is described as an easily degradable compound with over 90% elimination rate within two weeks, e.g. by Halling-Sorensen et al. (1998) or by Wiegel et al. (2003). On the contrary Ternes and Römbke (2005), Löffler et al. (2005) and Kreuzig et al. (2005) describe a strong tendency to sorption and persistence in the sediment and soil, which could amount to accumulation of paracetamol and its metabolites in the environment.

1.2.3 Diclofenac

Diclofenac (Figure 1.2) is one of the commonly employed non-steroidal anti-inflammatory drugs, often used to treat rheumatic pains, osteoarthritis and musculoskeletal injuries. It is a non-selective COX-1 and COX-2 inhibitor, causing in many cases adverse drug reactions, mostly gastrointestinal effects. Diclofenac is metabolized in the liver; only 1% of the drug is excreted unchanged via the kidneys. The first step in the metabolism is a hydroxilation reaction catalyzed by Cytochrome P450 monooxygenase isoenzymes (CYP). The major primary metabolite of diclofenac is 4'- hydroxy diclofenac (4'-OH diclofenac) catalyzed by CYP2C9 isoenzyme. Metabolites in smaller amounts are 3'-OH diclofenac, the production of which is catalyzed by CYP2C9 and 5'-OH diclofenac formed by CYP3A4, CYP2C19, CYP2C8 and CYP2C18 isoenzymes. In the second phase of the detoxification both diclofenac and its hydroxylated metabolites are conjugated with glucuronide and / or sulphate. The conjugates are mainly excreted via urine and in smaller amounts via bile (Kirchheiner et al. 2003). Both 4'-OH and 5'-OH diclofenac can be further oxidized to a p-benzoquinone imine, a highly reactive metabolite, causing oxidative stress and injuries.

In normal metabolism, all these intermediate metabolites are rapidly conjugated with glutathione and S-glutathionyl adducts can be further degraded and excreted from the body (Boelsterli, 2003).

1.2.4 Occurrence and fate of diclofenac in the environment

Diclofenac is one of the most frequently detected pharmaceuticals in WWTPs in µg/L concentration, and even the effluents seem to contain it often in high concentrations (Kasprzyk-Hordern et al. 2009; Daneshvar et al. 2010). Stülten et al. (2008) detected diclofenac, 4'-OH diclofenac (up to 1.7 µg/L) and 5'-OH diclofenac (up to 0.86 µg/L) in different wastewater treatment plant effluents in Germany. In many publications diclofenac was described to occur in ng/L to µg/L concentration in surface waters (Jux et al. 2002; Wiegen et al. 2003; Daneshvar et al. 2010). Diclofenac degrades mainly via phototransformation, since biological degradability is very low (Anderozzi et al. 2003).

There are some literature data about the ecotoxicological effects of diclofenac in the aquatic environment. Relatively low acute toxicity was found in case of invertebrates: Cleuvers (2004) obtained the half-maximum effective concentration (EC_{50}) in case of *Daphnia magna* in the range from 68 to 166 mg/L and in case of algal tests from 72 to 626 mg/L after 48 hour exposure. Ferrari et al. (2004) found similar EC_{50} values: 16 mg/L by

green alga, 14 mg/L by blue-green alga and about 22 mg/L by crustacean *Daphnia magna* and *Ceriodaphnia dubia* after 48 hour exposure. In rainbow trout 28 days exposure with 5 µg/L diclofenac leads to renal and kidney changes and collapse of the cellular compartmentation of the liver (Schwaiger et al. 2004; Triebskorn et al. 2004). These data refer to the acute effects of diclofenac; however their low but persistent occurrence in the environment will have mostly chronic rather than acute toxic effects. The toxicity of diclofenac was more drastic in case of vultures: in India and Pakistan the population of three vulture species collapsed because of fatal kidney failure caused by the drug. On the Indian subcontinent diclofenac is used widely in veterinary medicine to reduce inflammation caused by trauma and infectious disease by livestock. Poisoning occurred when the vultures fed on carcasses of treated animals (Oaks et al. 2004).

1.3 **Xenobiotic detoxification in plants: the three phase model**

Plants are exposed in many ways to pollutants in the environment; heavy metals, organic pollutants, xenobiotics from soil together with water and trace elements and air-borne gaseous pollutants pass into the root and leaf and affect the plant tissues. To avoid toxic effects, i.e. cell and tissue damages, plants have to metabolize them to a non-toxic form and sequester them from normal metabolic processes. These processes show similarities to animal xenobiotic detoxification in the liver. However an important difference from animal metabolism is the lack of an excretory pathway. All metabolites must be somehow stored in the plant tissues, in vacuoles or in cell walls. These processes can be divided in three phases: transformation (phase I), conjugation (phase II) and compartmentation (phase III). The reactions of the three phases and the involved enzyme systems have been first described by Shimabukoro (1976); the similarities of the metabolic pathways have been compared with animal metabolic patterns by Sandermann (1994) and summarized as the so called „green liver concept".

1.3.1 Phase I – Transformation

In the first metabolic phase the xenobiotic compounds are modified to prepare them for subsequent phase II and phase III processes. They are activated by oxidation, reduction or hydrolytic reactions that lead to greater water solubility and allow further conversions.

Hydrolytic reactions are de-esterification and amide hydrolysis catalyzed by esterases and amidases and have an important role in the bioactivation of xenobiotics. Reduction reactions can also be involved in the activation process, but they are less common than hydrolysis or oxidation. Reduction reactions in plants are aryl nitro reduction, where a nitro group on a phenyl ring is reduced to an amino group, deamination, where an amino group is removed, or photochemical reduction.

Oxidation reactions involve the loss of electrons and most of these reactions are catalyzed by Cytochrome P450 monooxygenases. Cytochrome P450 monooxygenases (CYP) are membrane bound haem proteins and members of a large enzyme superfamily present in microbes, fungi, plants and animals with diverse functions and broad substrate specificity. In the human metabolism they have a major function in drug metabolism. Above all they catalyze the biosynthesis of steroid hormones and cholesterol and vitamin D_3 metabolism (Werck-Reichhart and Feyereisen 2000). In plants CYP enzymes catalyze the biosynthesis of lignins, terpenes and alkaloids and play an important role in the secondary metabolism involved in the plant defense mechanism against microbial pathogens and insects. They are also involved in the detoxification of xenobiotics and pesticide tolerance (Schuler and Werck-Reichhart 2003). The first Cytochrome P450 enzyme gene was sequenced in 1990 (Bolwell et al. 1994). Presently 273 genes have been described from *Arabidopsis thaliana* alone (Nelson 2009).

1.3.2 Phase II – Conjugation

In the second phase of metabolism the activated xenobiotic compounds would be conjugated with glycosides, glutathione or amino acids. The main features of these conjugates are reduced biological activity, reduced toxicity, increased water solubility and reduced mobility.

Glucose conjugation results in plant *O*-, *S*-, and *N*-glycosides and malonyl-glucose conjugates, which are catalyzed by *O*-, and *N*-Glycosyltransferases and Malonyltransferases. Glycosyltransferases (GTs) are members of a super-family of more than 100 genes with a broad range of substrates, such as plant hormones, secondary metabolites and xenobiotics (Messner et al. 2003; Bowles et al. 2005). During the reaction GTs transfer activated sugar – mainly UDP-glucose or UDP-xylose – to various substrates, however the exact mechanism of the enzymes is not yet known (Ross et al. 2001).

Conjugation with amino acids is also a common reaction in plants. Mainly acidic insecticides, fungicides and herbicides have been metabolized this way. The most common amino acids involved in the conjugation processes in plants are aspartate and glutamate, but other amino acids are also involved, e.g. valine, leucine, phenylalanine and tryptophan. Amino acid conjugates are still biologically active compounds, nevertheless with strongly decreased mobility (Davidson et al. 1978; White et al. 1990).

Xenobiotics with electrophilic sites are conjugated into tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine) catalyzed by Glutathione S-Transferases (GSTs). GSTs are multifunctional enzymes located mainly in the cytosol and can also be found in membrane bound form (Pflugmacher et al. 2000; Dixon et al. 2002). GSTs have a dimeric structure composed of around 25-27 kDa size monomers and are present in plants mostly as heterodimers where the active sites of the subunits are distinct but related. Monodimeric GSTs with monomers containing identical active centers are also present in plants but are less frequent than the heterodimeric isoenzymes. The most common catalyzed GST reactions are nucleophilic aromatic substitutions, above all halogen removal, followed by Michael additions and epoxide ring opening reactions (Sheehan et al. 2001). These metabolites are more polar than the parent and cannot deliberately diffuse across membranes so that they have to be either sequestered in the vacuoles or transferred to the apoplast (Sandermann 1992; Edwards et al. 2000). The GST isoforms have a broad range of substrates among xenobiotic compounds and the role of GSTs is intensely investigated in the detoxification processes and described in numerous publications. However knowledge about their endogenous substrates is poor. There are only few reports about endogenous compounds in plant metabolism being conjugated with GSH such as medicarpin, caftaric acid or gibberellic acid (Edwards et al. 2000). Besides conjugation processes in the course of detoxification GSTs play a role in normal plant development and stress responses. Some GSTs have glutathione peroxidase activity (Dixon et al. 1998; Cummins et al. 1999), function as auxin carriers, transport anthocianin into the vacuole (Droog et al. 1995; Jiang et al. 2010) and play a role in cellular redox homeostasis and apoptosis regulation (Dixon et al. 2000; Sappl et al. 2009). They also showed induced activity during pathogen attack (Mauch and Dudler 1993; Lieberherr et al. 2003).

Members of the GST superfamily are present in eukaryotes and prokaryotes. The first classification of plant GSTs recognized three types of enzymes (Type I-II-III). However the permanently increasing number of identified new GSTs made a new classification necessary. The new organization of the GST superfamily includes the following classes:

Phi (GSTF) and Tau (GSTU) GSTs, which are plant specific isoenzymes, Theta (GSTT), Zeta (GSTZ), Lambda (GSTL) GSTs and Dehydroascorbate reductase (DHAR) (Dixon et al. 2010). The Zeta GSTs are found in plants and animals and the very heterogeneous Theta class is present in bacteria, yeast, plants as well as animals. Sigma, Alpha, Pi, Mu and Omega GSTs are present in animals and yeasts. Beta class GSTs have also been described in prokaryotes (Banerjee and Gowami 2009).

1.3.3 Phase III – compartmentation

Compartmentation of xenobiotic conjugates is unique to plants because unlike animals they lack a special excretion system; they have to remove the metabolites within their own limited cellular space. Hence their main processes in Phase III reactions are metabolism or further conjugation of Phase II products, sequestration of metabolites to the vacuole for storage and binding of metabolites into the cell wall region (Sandermann 1992; Van Eerd et al. 2003; Schröder et al. 2007).

Glucoside conjugates of herbicides can be further conjugated by the addition of a malonic acid residue from malonyl coenzym A in a reaction catalyzed by malonyl Co A transferase. The malonylation may enhance the vacuolar sequestration of the herbicide residue. In case of glutathione conjugates the formation of cysteine residues by the removal of glutamate and glycine groups is a typical secondary conjugation reaction.

Soluble residues like peptide or sugar conjugates are accumulated primarily in vacuoles. Xenobiotic metabolites are moved into the vacuole via ATP dependent carriers, GS-X pumps on the vacuole membrane (Theodoulou 2000). The carriers can recognize and transport mainly glutathione conjugates of xenobiotics and natural products (Martinoia 1993; Sandermann 1994; Coleman et al. 1997).

Insoluble or bound residues of xenobiotics are metabolites incorporated into the cell wall. They are coupled with lignin, starch, pectin, cellulose or xylan. Residues bound that way cannot be extracted with aqueous and organic solvents. Xenobiotics containing aromatic or heterocyclic rings are usually compartmented in this way (Sandermann et al. 1990).

1.3.4 Aims of the thesis

Besides classical organic pollutants and pesticides, pharmaceuticals and their residues are recognized as relevant environmental contaminants. The risk and the toxic effect of these chemicals for aquatic ecosystems are well described in the current literature, but information about the drug – plant interactions and metabolic pathways is scare. Therefore, the aim of the current work is the investigation of uptake and translocation of selected pharmaceuticals in two plant species, determination of the drug-induced defense responses and detoxification mechanisms in different plant parts and identification of the metabolites produced during the detoxification process.

The investigated pharmaceuticals are paracetamol and diclofenac, two of the most frequently used painkillers in the human medication. The uptake and metabolism of 1 mM paracetamol and 1mg/L paracetamol and diclofenac was investigated in Indian mustard (*Brassica juncea* L. Czern.) and broadleaf cattail (*Typha latifolia* L.) plants.

The main aims of this work are:

- Investigation of the uptake and translocation of the chemicals in plants and determination of the concentrations of pharmaceuticals in different plant tissues.

- Identification of the metabolic products and comparison of the detoxification mechanisms in plants with animal detoxification – the confirmation of the „green liver" concept.

- Investigation of the effect of pharmaceuticals on the main detoxification enzymes (Cytochrome P450 monooxygenases, Glutathione S-transferases, Glycosyl transferases and Peroxidases); their inducibility during the drug exposure.

- Determination of stress effects on plants; measuring the responses of the antioxidant enzyme system to the oxidative stress caused by paracetamol and diclofenac.

- Evaluation of the results in terms of ecotoxicological risk: are the human pharmaceutical residues able to enter the environment and reach the food chain via plant uptake and accumulation?

2 Materials and Methods

2.1 Plant material

2.1.1 *Brassica juncea* L. Czern. Var. 'Negro Caballo'

Figure 2.1 Indian mustard (*Brassica juncea* L. Czern.). The pictures originate from: http://de.wikipedia.org/wiki/Brassica_juncea

Kingdom *Plantae*, the Plants

- Division *Magnoliophyta*, the Angiosperms (flowering plants)
  - Class *Magnoliophyta*, Dicotyledons
  - Subclass *Dilleniidae*
    - Order *Capparales*
      - Family *Brassicaceae*, Mustard family
        - Genus *Brassica* L., Mustard

Brassica juncea L. Czern. (Indian- or brown mustard) is a 30-80 cm tall annual weed, a drought and heat tolerant close relative of canola (*Brassica napus*), developed as an alternative oilseed to canola.

Leaf mustard has been cultivated in Asia and Europe for thousands of years. The primary center of its origin being central Asia, the species now is widely distributed. Some cultivars are grown in India, China and Japan because of the high oil contents of the seeds. Others are grown for the leaves, roots, stems or flowers. In much of Europe, *B. juncea* has replaced *B. nigra* as the source of commercial mustard seed. Leaf mustard has escaped cultivation and become established as a weed in disturbed sites in North America.
Seeds germinate within 5 days after sowing at 20–25°C. Under good conditions plants grow rapidly and leaves are harvestable after 3 weeks when plants have developed 6–8 fully expanded leaves (http://www.floridata.com/ref/b/bras_jun.cfm).

Because of their high stress tolerance properties and good accumulation ability *B. juncea* is widely used in phytoremediation research, where mainly the heavy metal uptake and detoxification is investigated.

2.1.2 *Typha latifolia* L.

Figure 2.2 Common cattail (*Typha latifolia* L.). The pictures originate from:

http://upload.wikimedia.org/wikipedia/commons/d/d7/493_Typha_latifolia.jpg
http://de.wikipedia.org/w/index.php?title=Datei:Typha_latifolia_norway.jpg&filetimestamp=20050915200329

Kingdom *Plantae*, the Plants

- Division *Magnoliophyta*, the Angiosperms (flowering plants)
  - Class *Liliopsida*, the Monocotyledons
  - Subclass *Commelinidae*
    - Order *Typhales*, the Cattails
      - Family *Typhaceae*, Cattails
        - Genus *Typha*, Cattails

Typha latifolia L. (Common Cattail, or Broadleaf Cattail) is a common perennial marsh or wetland plant found in the northern hemisphere and Australia. Plants are typically 1.5-3 meters high with 2-4 cm wide leaves and stems of the plant bear long flower spikes with an upper male staminate section and a lower female pistillate section. The root system produces thick starchy rhizomes, which are producing vegetative colonies.

Cattail is an emergent aquatic plant, rooted in the soil or sediment, but stems, leaves and flowers grow above the surface. It is adapted to a wide range of soil and water conditions, even hypoxia: the stems contain aerenchyma (air spaces) that allow oxygen transport down through the stem to the roots.

Vegetation cover of *Typha* plants plays an important ecological role in the aquatic ecosystems; they provide habitat for many lakeside species, filter the runoff as it flows into the water bodies from excessive amounts of nutrients or pollutants and therefore play a role in natural water cleaning. The plants also provide direct benefits to humans; many parts of the plant are edible and the leaves provide building material or versatile raw material for the production of everyday objects like furniture and mats (http://www.newworldencyclopedia.org/entry/Typha). *Typha* is one of the most frequently used plants in constructed wetlands, because of its great filter capacity and tolerance against pollutants.

2.1.3 *Brassica* experimental setup

Brassica juncea seeds were obtained from the Hungarian Research Institute for Medical Plants (Budakalász, Hungary). Seeds were germinated in perlite irrigated with full strength Hoagland medium under greenhouse conditions. After four weeks the plants were transplanted in plastic pots and grown in full strength Hoagland medium. Each pot was filled with 3 L medium and contained 4 plants. Nutrient solution was changed once in every week.

Eight weeks old healthy plants were treated with paracetamol and diclofenac added to the nutrient solution for one week. Root and leaf samples were collected 24, 72, and 168 hours after treatment, immediately frozen in liquid nitrogen and stored at -85°C till sample preparation.

To determine abiotic degradation of the active compounds the drug concentrations were monitored in the growth medium of the plants and in growth medium without plants, using the same treatment conditions.

Table 2.1 Pharmaceutical treatments of *Brassica juncea*.

Compound	Concentration	Exposure time	Sampling time
paracetamol	1 mM (151 mg/L)	1 week	24, 72, 168 h
	1 mg/L	1 week	24, 72, 168 h
diclofenac	1 mg/L	1 week	24, 72, 168 h

Table 2.2 Composition of Hoagland liquid culture medium.

Macronutrients	Concentration	Micronutrients	Concentration
$Ca(NO_3)_2 \cdot 4H_2O$	2 mM	$CuSO_4 \cdot 5H_2O$	0.48 µM
FeNa-EDTA	0.1 mM	H_3BO_3	48 µM
KH_2PO_4	1 mM	$MnCl_2 \cdot 4H_2O$	9 µM
KNO_3	0.5 mM	$Na_2MoO_4 \cdot 2H_2O$	0.4 µM
$MgSO_4 \cdot 7H_2O$	2 mM	$ZnSO_4 \cdot 7H_2O$	0.5 µM

2.1.4 *Typha* experimental set up

18 month old *Typha latifolia* rhizomes were obtained from a plant breeder (Jörg Petrowsky, Eschede). After cleaning the roots and rhizomes the plants were transplanted in 3 L plastic pots in perlite and irrigated with 10 fold diluted Murashige & Skoog medium (Duchefa Biochemie B. V., Netherlands). After a 2 month growing period the plants were transplanted in to Murashige & Skoog medium (each plant in 3 L Medium) and grown 2 more weeks to acclimatize to the hydroponic conditions before starting the experiments. Nutrient solution was changed once every week.

Plants were treated with paracetamol and diclofenac added to the nutrient solution. Root and leaf samples were collected 24, 72, 168 hours and 30 days after treatment, immediately frozen in liquid nitrogen and stored at -85°C till sample preparation.

Table 2.3 Pharmaceutical treatments of *Typha latifolia.*

Pharmaceutical	Concentration	Exposure time	Sampling time
paracetamol	1 mM (151 mg/L)	1 week	24, 72, 168 h
	1 mg/L	1 month	24, 72, 168 h and 30 days
diclofenac	1 mg/L	1 month	24, 72, 168 h and 30 days

Table 2.4 Composition of MS liquid culture medium (Duchefa Biochemie B. V.).

Macronutrients	Concentration	Micronutrients	Concentration
$CaCl_2$	2.99 mM	$CoCl_2 \cdot 6H_2O$	0.11 µM
KH_2PO_4	1.25 mM	$CuSO_4 \cdot 5H_2O$	0.1 µM
KNO_3	18.79 mM	FeNa-EDTA	0.1 µM
$MgSO_4 \cdot 7H_2O$	1.5 mM	H_3BO_3	100 µM
NH_4NO_3	20.61 mM	KI	5 µM
		$MnSO_4$	100 µM
		$Na_2MoO_4 \cdot 2H_2O$	1.03 µM
		$ZnSO_4 \cdot 7H_2O$	29.9 µM

2.1.5 Short-term pharmaceutical uptake experiment from culture medium

In the experiment 50 mL falcon tubes were filled with 30 mL Murashige & Skoog medium containing 1 mg/L and 10 mg/L paracetamol or diclofenac. In one series of tubes 2 g *Typha latifolia* roots cut from 2 month old plants were placed and stored at room temperature in dark till sampling. Falcon tubes were filled with the medium containing the same pharmaceutical concentration without plant material as control to detect the possible depletion of the active compound by adsorption to the tube wall. The medium and the plant material were sampled after 1, 2, 4, 6, 8 and 24 hours of exposure, immediately frozen in liquid nitrogen and stored at -85°C till sample preparation.

2.2 **Extraction of the pharmaceuticals and metabolites**

2.2.1 Extraction of paracetamol and metabolites from plant tissues

For the extraction of paracetamol and its metabolites, 0.5 g root and leaf tissues were homogenized under liquid nitrogen and extracted in 1 mL 0.1 M HCl. The samples were mixed properly and incubated on ice for 20 minutes. The mixing of the samples was repeated several times during the incubation of the extract. The samples were centrifuged at 12,000 rpm and 4°C for 15 minutes. After centrifugation 700 µL of supernatant were collected and loaded on SPE columns (Phenomenex Strata-X, 30 mg/L sorbent mass). Acetanilide was added to the supernatant prior to the solid phase extraction as an internal standard at a concentration of 10 µg/L.

2.2.2 Extraction of diclofenac and metabolites from plant tissues

Because of the moderate solubility of diclofenac in water 1 mL 0.1 M HCl/acetonitrile (50/50, v/v) mixture was used for the extraction under the same conditions as above. Before the solid phase extraction 5-(p-Methylphenyl)-5-phenylhydantoin was added to the supernatant as an internal standard at a concentration of 10 µg/L.

2.2.3 Solid phase extraction (SPE)

To clear up the active compounds and their metabolites from the plant extract a solid phase extraction was carried out by Strata-X Reversed Phase columns (Strata-X 33 µm, Polymeric Sorbent). Standard protocol from Phenomenex for Strata-X SPE columns was used; after preconditioning with 1 mL of 100% methanol and 1 mL of water (MiliQ) 700 µl of extract was loaded on the columns. After loading the extracts, columns were washed with 1 mL of 5% methanol to remove impurities. Analytes were eluted with 700 µl acetonitrile/methanol (50/50, v/v) under gravity with a flow rate of less than 1 mL/min. The samples were fully vaporized to a solid state, stored at 4 °C and before the LC-MS analysis dissolved in 200 µL of 3% acetonitrile with 0.1% formic acid.

2.3 **LC-MS analysis of the pharmaceuticals and metabolites**

LC-MS analysis was carried out with an ion trap mass spectrometer (Varian 500-MS, Varian, Darmstadt) coupled to a ProStar 210 HPLC module (Varian, Darmstadt) with a ProStar 410 auto sampler (Varian, Darmstadt). The electrospray ionization ion source was operated in positive ion mode. Analytes were separated using a C18 polar end-capped Hydro-RP column with dimensions of 50mm x 2mm and 4 µm particle size (Phenomenex, Aschaffenburg). Mobile phases consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) run at a constant flow rate of 0.3 mL/min. All solvents used for LC-MS were of the highest grade available (Carl Roth, Karlsruhe). The solvent gradient was initially held at 3% solvent B for 2 min, increased linearly to 95% solvent B over another 8 min, kept at 95% solvent B for 2.5 min and dropped to 3% solvent B over 2 min where it was held constant at 3% solvent B for a further 4.5 min before the next sample injection to equilibrate the column.

The electrospray settings were optimized to obtain the maximum sensitivity for the analytes (Table 2.5). MS/MS spectra were obtained by collision-induced dissociation using helium as collision gas.

Table 2.5 Optimized electrospray settings of the HPLC-MS method to determine the pharmaceutical compounds and their metabolites.

Electrospray parameters:	Settings:
Capillary voltage	40 V
Needle voltage	4000 V
Sprayshield voltage	600 V
Nebulizer gas pressure	50 psi (3,45 bar)
Drying gas pressure	30 psi (2,1 bar)
Drying gas temperature	300 °C

2.3.1 Paracetamol and its metabolites in plants

To quantify the paracetamol amount taken up by the plants and determine its metabolites the following mass transitions were measured: for internal standard (acetanilide) 136→94; paracetamol 152→110; paracetamol glutathione conjugate 457→328; and paracetamol glucosyl conjugate 314→152→110.

Peak areas from each of the transitions were obtained and ratios of the analytes to internal standard were calculated for each sample. Concentration of free paracetamol was determined using a calibration curve with external standard (Fig. 2.3).

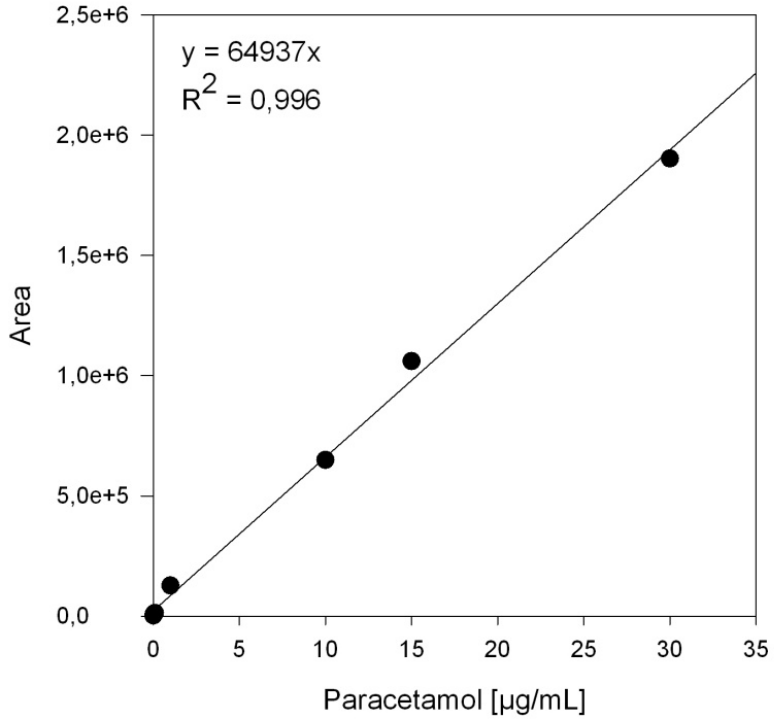


Figure 2.3 Calibration curve for paracetamol quantification in medium and plant samples. Standards were prepared in 3% acetonitrile with 0.1% formic acid.

2.3.2 Diclofenac and their metabolites in plants

The determination of diclofenac and its dominant metabolites were obtained by measuring the following mass transitions: internal standard (5-(p-Methylphenyl)-5-phenylhydantoin) 267→196; diclofenac 296→278; OH-diclofenac 312→294; diclofenac-glucopyranose 474→312 and OH-diclofenac -glutathione 617→342.

Peak areas from each of the transitions were obtained and ratios of the analytes to internal standard were calculated for each sample. Concentration of free diclofenac was determined using a calibration curve for the external standard (Fig. 2.4).

2.3.3 Detection of pharmaceuticals in medium samples

For the determination of paracetamol and diclofenac concentration in nutrient medium, 1.5 mL of growth medium was filtrated with 0.45 µm pore size syringe filters (Rotilabo-syringe filters, Carl Roth, Karlsruhe) and directly analyzed with LC-MS without further cleanup.

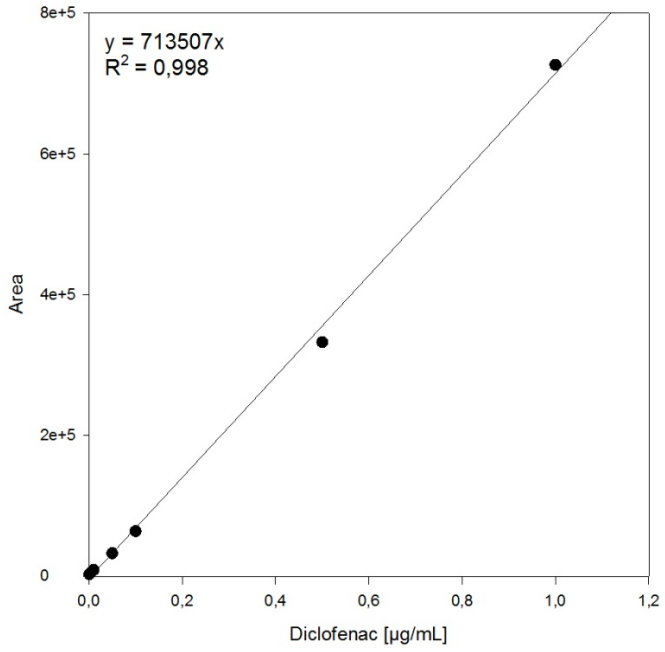


Figure 2.4 Calibration curve for diclofenac quantification in medium and plant samples. Standards were prepared in 3% acetonitrile with 0.1% formic acid.

2.4 **Preparation of microsomal enzyme fraction from plants**

The extraction of the microsomal fraction was performed with some modification as described by Page and Schwitzguebel (2009). Frozen plant material (10 g) was ground in a blender for 30 s, dissolved in 50 mL pre-cooled 0.1 M Na phosphate buffer (pH 7.4) and stirred for 15 min on ice. The extraction buffer contained 250 mM saccharose, 1 mM EDTA, 40 mM ascorbic acid, 1 mM PMSF and 10 mM DTE. The homogenate was filtered on Miracloth and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was collected and ultra-centrifuged at 100,000 x g for 1 h at 4 °C. After ultracentrifugation the supernatant was collected, immediately frozen in liquid nitrogen and stored at -83°C for further enzyme extraction. The pellet containing the microsomes was dissolved in 1 mL 0.1 M Na phosphate buffer pH 7.4 and homogenized in a glass potter. The microsomes were stored at -83°C. Concentration of proteins in the extract was measured using Bradford assay.

2.5 **Preparation of cytosolic enzyme fraction from plants**

The extraction of the enzymes from the plant cytosolic extract was performed as described by Schröder and co-workers (Schröder at al. 2005) with some modification. The enzymes were extracted from the supernatant collected during the microsomal enzyme extraction. The samples (stored on -83°C) were unfrozen and aliquoted in glass beakers (20 mL). The proteins in the supernatant were precipitated by stepwise addition of solid ammonium sulphate. In the first step the salt saturation was increased up to 40% by slowly adding fine grounded $(NH_4)_2SO_4$. The samples were stirred on ice for 30 min with a magnetic stirrer, after that the extracts were centrifuged at 4°C and 20,000 x g for 30 min. The supernatants were collected and in a second step the salt saturation was increased up to 80% and stirred another 30 min on ice. The samples were centrifuged again at 4°C and 20,000 x g for 30 min. After the second centrifugation the supernatants were discarded and the pellets were re-suspended in 2 mL of 25 mM Tris/HCl buffer (pH 7.8). The extracts were desalted by passing them through PD-10 gel filtration columns (Pharmacia, Freiburg) and eluated in 3.5 mL 25 mM Tris/HCl buffer (pH 7.8). The samples were aliquoted and stored at -83°C. Concentration of proteins in the extract was measured using Bradford assay.

2.6 **Determination of Glycosyltransferases (EC 2.4.-.-) activity**

O-Glycosyltransferase activity was determined using the method described by Messner et al. (2003). In the reaction mixture 0.1 mM substrate (quercetin, kaempferol or TCP) was incubated with 2 mM uridine diphosphate glucose (UDPG), 3.125 mM N-Nitrophenyl-D-Glucopyranoside and 3,125 mM Salicin in 200 mM Tris/HCl buffer (pH 7.3) containing 2 mg $MgCl_2$ (buffer A). The reaction was started by adding 100 µL cytosolic enzyme extract (Table 2.6). After 30 min incubation at 30°C the reaction was stopped by addition of 10 µL H_3PO_4 and cleared by centrifugation at 15,000 x g for 2 min. The supernatant was diluted 1:4 (v/v) with HPLC solvent A (water, 0.1% TFA). Reverse phase HPLC was performed using a Varian Pro-Star M215 HPLC system and a C18 Prontosil Spheribond column (Bischoff Chromatography, Leonberg). Mobile phases consisted of 0.1% aqueous trifluoroacetic acid (solvent A) and acetonitrile with 0.1% TFA (solvent B) run at a constant flow rate of 0.85 mL/min (Table 2.7).

Table 2.6 Reaction mixture composition of the GT assay.

Substance [mM]	Concentration in enzyme-assay [mM]	Volume [µl]
Substrate-Solution [2]	0.1	10
UDPG [20]	2	20
Buffer A	-	50
Protective Substances:	-	-
4-NPG [25]	3.125	10
salicin [25]	3.125	10
Enzyme extract		100
Total volume		200

Table 2.7 HPLC solvent gradients used in the GT assay.

Time [min/sec]	Solvent A [%]	Solvent B [%]
0	92	8
8	0	100
9:30	0	100
12:30	92	8
15	92	8

2.7 **Spectrophotometric enzyme assays**

The enzyme activity assays were carried out using a 96-well plate reader SECTRAMax PLUS 384 spectrophotometer (Molecular Devices, Ismaning) with the data analyzing software SOFTmax PRO 4.6.

96-well plates from Nunc (Brand, Wertheim) were applied for measuring in the visible light spectrum (390-750 nm); for measuring in the UV spectrum specific plates from Greiner (Greiner, Frickenhausen) were used. In the standard kinetic tests OD changes were measured in 15 sec intervals for 5 min at room temperature. The samples were measured using three technical replicates. A reaction mixture without enzyme extract was used as blank; the enzyme activities are expressed in μkat (μmol/sec).

2.7.1 Glutathione-S transferases, GST (EC 2.5.1.18)

The GST activity was determined by using different substrates, which cover the activities of different isoforms of the enzymes. In the reaction mixture (RM) GSH and substrates were mixed in a buffer. The reaction was started by mixing 190 μL RM with 10 μL enzyme extract in the 96 well plates. The formation of the substrate-GSH conjugate was recorded using the specific wavelength as give in the literature. The concentration of substrates, GSH, the buffers, wavelengths and substrate specific extinction coefficients are summarized in Table 2.8.

Table 2.8 GST substrates and their concentrations in the reaction mixture.

Substrate	Concentration [mM]	GSH conc. [mM]	Buffer	Wave-length [nm]	Ext. coefficient [$mM^{-1}cm^{-1}$]
1-chloro-2,4-dinitrobenzene (CDNB)	1	1	Tris/HCl 0.1M; pH 6.4	340	9.6
1,2-dichloro-4-nitrobenzene (DCNB)	1	1	Tris/HCl 0.1M; pH 7.5	345	8.5
p-nitrobenzylchlorid (pNBC)	0.5	1	Tris/HCl 0.1M; pH 6.4	310	1.8
p-nitrobenzoylchlorid (pNBoC)	0.5	1	Tris/HCl 0.1M; pH 6.4	310	1.9
p-nithrophenylacetate (pNpa)	0.2	1.2	KPP 0.1M; pH 7.0	400	8.79
fluorodifen	0.3	0.5	Tris/HCl 0.1M; pH 7.5	400	3.1

2.7.2 Peroxidase, POX (EC 1.11.1.7)

Peroxidase activity was measured with the method proposed by Roy and Hänninen (1994) using guaiacol (2-methoxyphenol) as substrate. The RM contains 190 µM H_2O_2 and 68 µM guaiacol in 50 mM Tris/HCl buffer (pH 6). The reaction was started by mixing 190 µL RM with 10 µL enzyme extract in the 96 well plates. Thereafter the formation of tetraquaiacol (ε = 26.6 $mM^{-1}cm^{-1}$) was recorded at 420 nm wavelength.

2.7.3 Ascorbate peroxidase, APOX (EC 1.11.1.11)

APOX activity was assayed by recording the oxidation of ascorbic acid at 290 nm (Vanacker et al. 1998). RM contains 1 mM H_2O_2 and 250 µM ascorbic acid in 55.56 mM KH_2PO_4 (pH 7). The reaction was started by mixing 180 µL RM with 20 µL enzyme extract in the 96 well plates and the decrease of ascorbic acid concentration was recorded (ε = 2.8 $mM^{-1}cm^{-1}$).

2.7.4 Catalase, CAT (EC 1.11.1.6)

CAT was assayed by the method of Verma and Dubey (2003). The reaction mixture contained 53 mM H_2O_2 in 100 mM KH_2PO_4 (pH 7). 140 µL RM was mixed with 10 µL enzyme extract and the decrease of H_2O_2 concentration ($\varepsilon = 0.036\ mM^{-1}cm^{-1}$) was recorded at 240 nm.

2.7.5 Glutathione reductase, GR (EC 1.6.4.2)

GR activity was measured as described by Zhang and Kirkham (1996). The RM contains 1 mM oxidized glutathione (GSSG) and 2 mM NADPH in 100 mM Tris/HCl buffer (pH 7.5) with 0.1 mM EDTA. The reaction was started by mixing 190 µL RM and 10 µL enzyme extract. In the reaction the glutathione reductase reduces GSSG to GSH using NADPH. The decrease of NADPH concentration ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$) was recorded at 340 nm.

2.7.6 Protein content determination

Protein contents were determined by the method of Bradford (1976) using Coomassie Brilliant Blue G250 dye. 10 µL enzyme extract was added to 200 µL ten-fold diluted Coomassie Blue dye. The OD was measured at 595 nm after 10 min. incubation at room temperature. The protein content was calculated using a BSA calibration curve and expressed in mg/mL.

2.8 **RNA isolation and quantification**

Total RNA was used to quantify the expression of specific genes using quantitative PCR. RNA isolation was carried out from control and treated *Brassica juncea* root and leaf tissues using Bio&SELL RNA Mini Kit (Bio&SELL, Nürnberg), designed for fast and efficient isolation of total RNA from different tissues. All of the required solutions were supplied by Bio&SELL. The extraction procedure was carried out according to the protocol provided by Bio&SELL. The protocol description in brief: 20 mg frozen plant tissue was grounded to a fine powder under liquid nitrogen using mortar and pestle. The samples were lysed using lysis buffer, then the lysate was applied to a spin column to remove genomic DNA and inactivate RNases. After the binding and washing steps the pure RNA was eluted in the final step with RNase-free water. The samples were stored at

-83°C. The amount of isolated RNA was quantified with a Nano Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, München) at 260 nm. The purity of the RNA was determined by the ratio of absorbance at 260/280 nm and 260/230 nm. Pure RNA should give a A_{260}/A_{280} value of approximately 2.0 and the ratio A_{260}/A_{230} should be approximately 2.2.

2.9 **Reverse transcription (RT-PCR)**

First strand DNA was synthesized from 1 µg of total RNA in 20 µL reaction volume using the RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, St. Leon Roth, Germany) as per the manufacturer's instructions. A volume of 0.25 µl (25 pmol) oligo (dT)18 primer, 1 µL 10 mM dNTP Mix and 15 µL nuclease-free water were added to the 1 µg RNA (volume was calculated for each sample). The samples were mixed gently and incubated in the thermal cycler at 65° C for 5 min and immediately chilled on ice. A volume of 4 µL 5x RT buffer and 1 µL RevertAid Premium Enzyme Mix (contains M-MuLV RT and RiboLock RNase inhibitor) were added into the tubes, mixed gently and centrifuged. The samples were incubated in the thermal cycler for 10 min at 25°C followed by 30 min at 50°C. Finally the reaction was terminated by heating at 85°C for 5 minutes. The tubes were stored at -20°C.

2.10 **Quantitative real-time PCR (Q-PCR)**

Monitoring of the expression rate of GST genes was performed with quantitative real time PCR using a 7300 Real Time PCR System (Applied Biosystems, Carlsbad, California). All reactions were performed in triplicate using SYBR Green PCR Master Mix (Fermentas). The PCR conditions consisted of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing reaction at 60°C for 1 min. A melting curve was generated at the end of each PCR cycle. Non template controls (NTC) were measured for each primer pairs as negative control. The data analysis was performed using the 7300 System SDS software (Applied Biosystems). Quantification was assessed by measuring the threshold cycle values (C_T); the relative gene expression rates were calculated using Relative Expression Software Tool (REST©) Software.

2.11 **Primer design for Q-PCR**

Gene specific primers were designed for the genes of *Brassica* GSTU5, GSTU19, GSTU24, GSTF6 and for the housekeeping genes 18S rRNA, actin and GAPDH (Table 2.9). The sequences of the genes were found in the NCBI genomic database. To detect similarities the sequences were aligned using the Clustal W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The primers were designed using the Primer3 (v. 0.4.0) software and purchased from the company Eurofins MWG Operon (Ebersberg).

Table 2.9 Primer sequences for Q-PCR

Gene	Forward primer sequence	Reverse primer sequence
GST-F6	AATGGCAGCTATCAAAGTTTTCG	TGGAGGGTGAGGACGACTCT
GST-U5	GCCGAGCAGACAAGAGAACT	CAAATCCAAAAACCCGACAC
GST-U19	TCGGACCAGTATGTTTCAAGTC	ATCTATTCCCTTATCTGCTTTCACC
GST-U24	AACCCCTTCCTTCCTTCTGA	CGCCGTAACATTCACCTTTT
18S rRNA	TCGGAAGTAGTGCTGCAATG	GGGATTCTGCAATTCACACC
Actin	GCATGAAGATCAAGGTGGTG	TCTGTTGGAAAGTGCTGAGG
GAPDH	TTGTTTCCAACGCTAGTTGC	CAATTCCAAACCTGTCGTTG

3 Results

The effects of human pharmaceutical residues in the environment have been intensely investigated in the last years and focused first of all on invertebrates and higher organisms in the aquatic ecosystems. There are few publications about human and veterinary drug uptake experiments in higher plants, but no information about the metabolism and the effects within plants (Boxall et al. 2006; Redshaw et al. 2008; Eggen et al. 2011).

The present work has the goal to investigate the fate of human pharmaceuticals in plants and give a complex picture of the fate of these pollutants from uptake to detoxification and stress reactions of plants. In chapter 3.1 and 3.2 the results of paracetamol uptake and metabolism are described in the two experimental plant species *Typha latifolia* and *Brassica juncea*. Diclofenac uptake and metabolism for these species is described in chapters 3.3 and 3.4.

3.1 **Paracetamol uptake and detoxification in *Typha latifolia***

3.1.1 Uptake and translocation of paracetamol

To evaluate paracetamol detection in the samples first pure paracetamol was analyzed. Fragmentation of 152 → 110 *m/z* ion for paracetamol was detected using the tandem MS (MS/MS) mode of the LC-MS. This fragmentation occurs due to the loss of an acetyl fragment of the *N*-acetyl group and produces a primary ammonium ion with 110 *m/z*. The retention time was 3.8 min (Fig. 3.1).

1 mM paracetamol exposure

In the first experiments plants were exposed to 1 mM of paracetamol (equates 151 mg/L) and the uptake was measured after 24, 72 and 168 hours of exposure in root and leaf tissues of cattail plants.

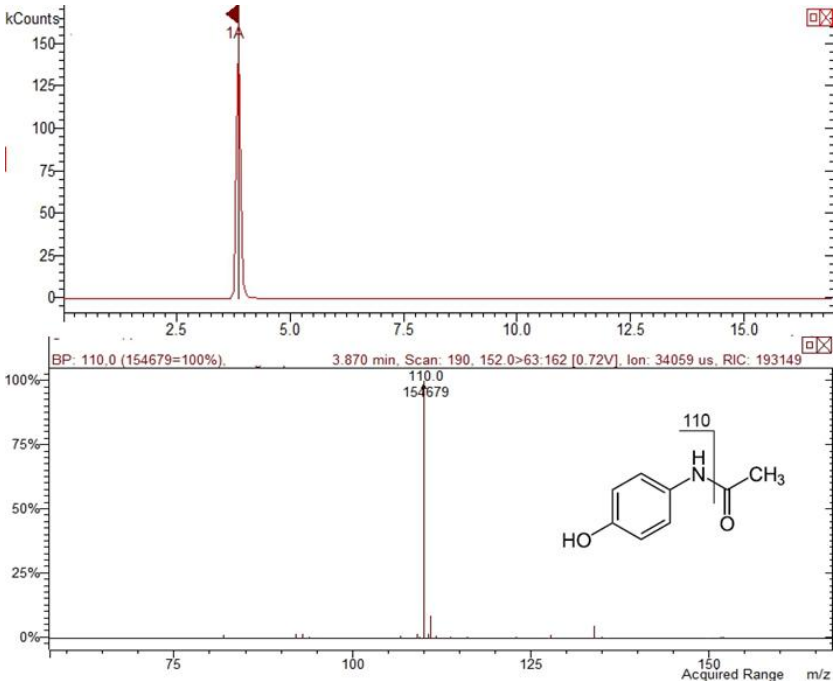


Figure 3.1 LC-MS/MS chromatogram and spectra of a paracetamol standard $(M+H)^+$ *m/z* 152 using the positive ionization mode.

Already after 24 hours paracetamol was detected in the roots (37 µg/g FW) and in the leaves (6.6 µg/g FW) of *Typha* plants. It reached its maximum after 72 hours with 87 µg/g FW in the roots and 28 µg/g FW in the leaves respectively. This indicates a rapid uptake and the presence of transport mechanisms between the plant tissues. One week after treatment the concentration of paracetamol decreased in all tissues, in the root however, after a moderate decrease, still 55.3 µg/g FW was measured (Fig. 3.2).

1 mg/L paracetamol exposure

The experiment with 1 mg/L treatment concentration was carried out for 30 days; tissues were sampled after 24, 72, and 168 hours and at the end of the experiment, after 30 days of exposure. The uptake course into the root shows similarities to the high concentration experiment. The maximum uptake is already reached after 24 hours (0.11 µg/g FW), followed by decreasing paracetamol content during the next six days of exposure. After 30 days about 50% of the initial concentration was detected (0.068 µg/g FW) in the root tissues. The transport to the upper parts of the plants was significantly higher; at the end of the first week 0.077 µg/g FW of paracetamol (about 78% of the total amount) was present in the leaf tissues. After 30 days the concentration decreased, but still about 30% of the drug was present in the shoot tissues (Fig. 3.3).

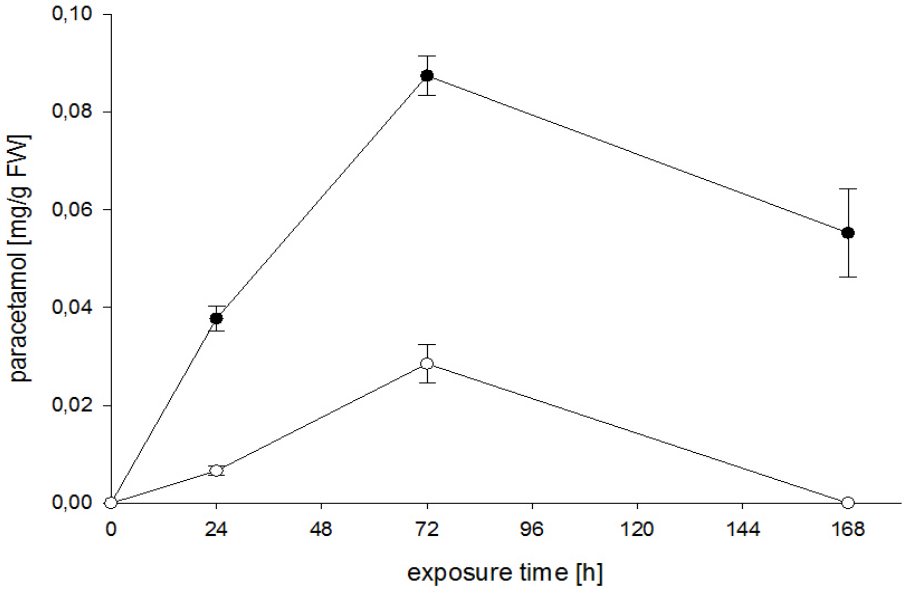


Figure 3.2 Paracetamol uptake and transport in *Typha* root (●) and leaf (○) tissues after 1 mM pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

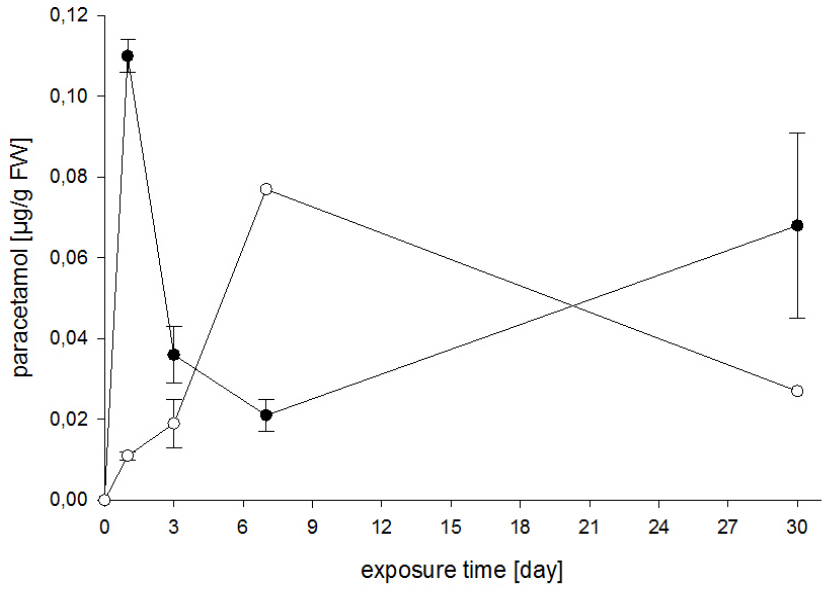


Figure 3.3 Paracetamol uptake and transport in *Typha* root (●) and leaf (○) tissues after 1 mg/L pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

3.1.2 Short-term uptake of paracetamol from culture medium

Paracetamol concentrations were determined with HPLC-MS measurements in the incubation medium in the presence of *Typha latifolia* root tissue and in a control medium without plant tissues as well as in the plant root tissues. Two falcon tubes were filled with 30 mL liquid medium containing 1 mg/L and 10 mg/L paracetamol.

The paracetamol content strongly decreased in the medium incubated with plant roots. After two hours less than 50% was detected, but after 4 hours no further decrease was observed (Fig. 3.4, dotted line). In parallel, uptake of paracetamol was detected in the root samples (Fig. 3.4, dashed line), but the concentration in the plant tissues did not match the missing concentration in the medium. The difference might be due to adsorption onto the root surface. This assumption is confirmed by the results of the control samples where no adsorption effect was observed in the absence of plant tissues (Fig. 3.4 solid line); the paracetamol concentration did not decrease in the medium. Table 3.1 represents the calculated surface adsorption and the uptake rates in the root segments.

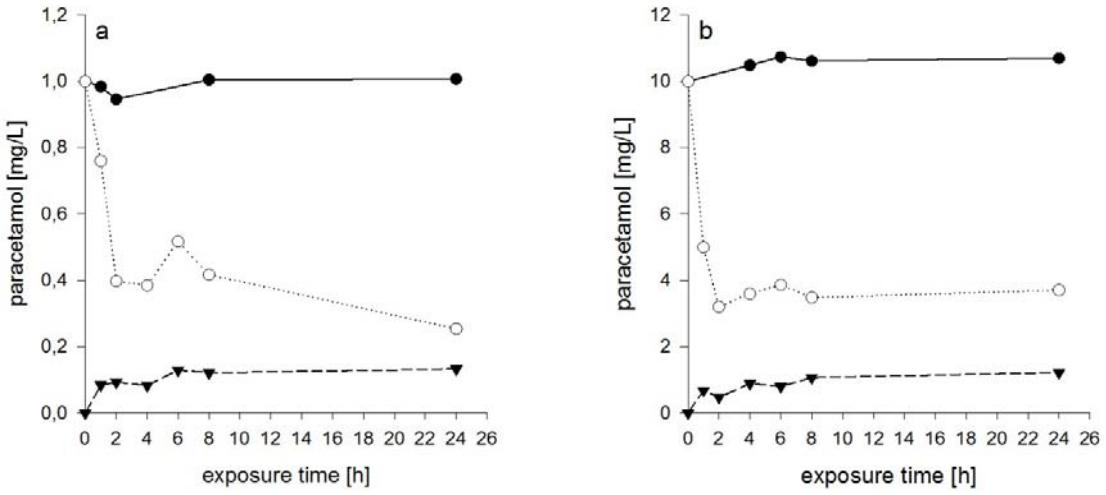


Figure 3.4 Paracetamol concentration changes in medium containing 1 mg/L paracetamol (a) and 10 mg/L paracetamol (b) in the absence of root material (solid line ●), in the presence of root material (dotted line ○) and in the root tissues (dashed line ▼).

Table 3.1 Fate of paracetamol from the culture medium due to surface adsorption and uptake by *Typha* roots during 24 hours. N. B.: The adsorption could not be calculated on a surface basis because roots did not represent ideal cylindrical form.

time	1 mg/L			10 mg/L		
	surface adsorption (mg)	uptake (mg/g FW)	uptake rate (mg/min/g FW)	surface adsorption (mg)	uptake (mg/g FW)	uptake rate (mg/min/g FW)
1 h	0.14	0.084	0.0014	6.65	0.678	0.011
4 h	0.71	0.083	0.0003	6.99	0.892	0.004
6 h	0.55	0.129	0.0004	6.07	0.803	0.002
8 h	0.51	0.121	0.0003	7.07	1.067	0.002
24 h	0.69	0.132	0.0001	6.77	1.216	0.001

3.1.3 Identification of paracetamol metabolites in *Typha latifolia*

The metabolic products of paracetamol in mammals are well investigated and described in the literature, but there is little information about detoxification in other organisms. The first paracetamol conjugates in plants were described by Huber et al. (2009) in a hairy root culture of horseradish, where two metabolic pathways were identified that result in the formation of a glutathione and a glucose conjugate. The same conjugates were found in cattail plants exposed to different paracetamol concentrations.

Conjugation with sugar is one of the main xenobiotic detoxification processes in plants. Huang et al. (2006) described paracetamol conjugation with glucose in filamentous fungi; Huber et al. (2009) identified paracetamol glucoside conjugates in plant tissues. The fragmentation of the pseudo molecular ion *m/z* 314 → 152 was detected as described by Huber (2011). *O*-glucosides show the typical loss of 162 Da due to the cleavage of dehydroglucose during MS/MS fragmentation, resulting in the pseudo molecular ion of the parent compound paracetamol itself (Fig. 3.5). The presence of paracetamol glycopyranoside was further confirmed by incubation with *β*-glucosidase, which leads to the disappearance of the conjugate.

Conjugation with glutathione plays a major role in xenobiotic detoxification in plants: a broad range of xenobiotic compounds is metabolized in to respective conjugates (X-GS) and finally transported to the cell vacuoles. In case of paracetamol the conjugation with glutathione is already described in mammalians. The paracetamol-GS conjugate can be identified due to the detection of a molar mass transition of 457 → 328 *m/z* ion (Fig. 3.5). The product ion is formed by the loss of the 129 Da glutamic acid residue of glutathione resulting in the formation of a fragment of *m/z* 328 (Mutlib et al., 2000).

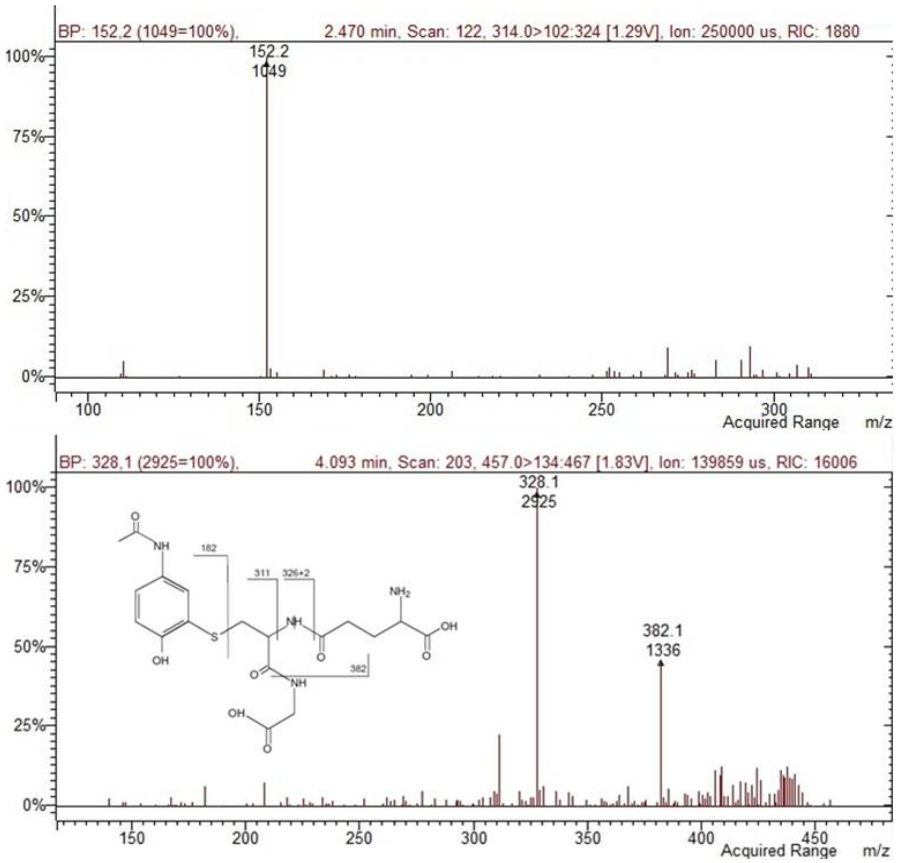


Figure 3.5 MS/MS spectra of the paracetamol glycopyranoside $(M+H)^+$ *m/z* 314 and paracetamol-GS $(M+H)^+$ *m/z* 457 conjugates in *Typha latifolia* root tissues using positive ionization mode.

1 mM paracetamol exposure

Glutathionyl and glycoside conjugates were identified in plants exposed to 1 mM paracetamol in root and shoot tissues. During the first 72 hours nearly the same amount of both metabolites was detected in the root tissues. One week after treatment the relative amount of paracetamol-GS conjugate was nearly 6 fold higher, whereas the amount of the pyranoside conjugate remained constant (Fig. 3.6 a). The relative amount of GS-conjugate in the shoots after 24 and 72 hours is almost the same as in root tissues. Compared to the root no significant increase of the conjugate amount was detected at the end of the experiment (Fig. 3.6 b). The paracetamol-glucopyranoside conjugate in the shoots could only be detected after 72 hours of exposure and was completely depleted after one week. This time dependent formation of the pyranoside conjugate correlates with the presence of free paracetamol in the shoot tissues, which also showed depletion after one week (Fig. 3.2) and indicates metabolic activity in both tissues.

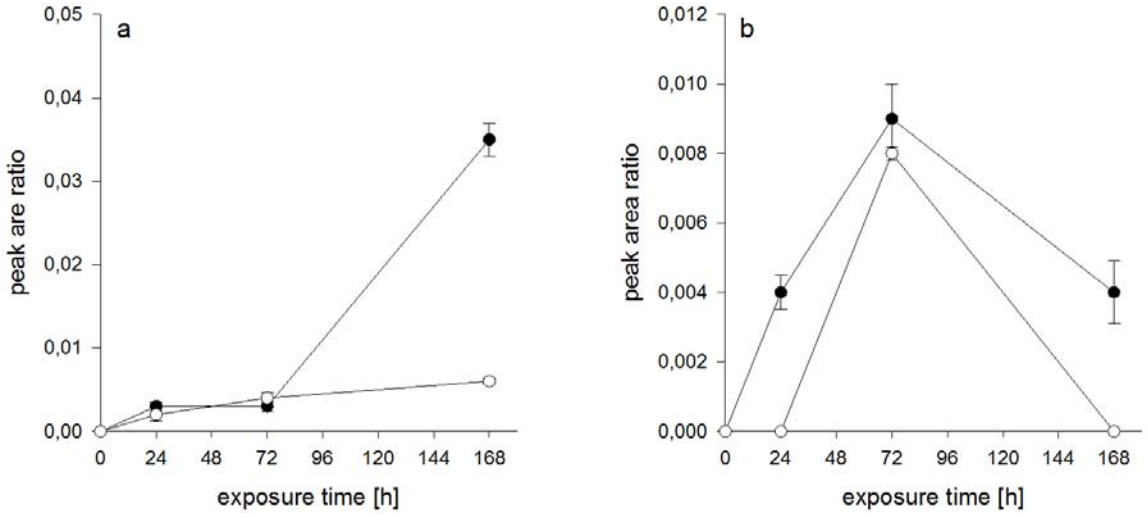


Figure 3.6 Relative amounts of paracetamol-GS (●) and paracetamol-glucopyranoside (○) conjugates in *Typha* root (a) and shoot (b) tissues after 1 mM pharmaceutical exposure.

1 mg/L paracetamol exposure

After 24 hours of exposure to 1 mg/L paracetamol only the conjugation product with glutathione was clearly detected in the root tissues. It reached a maximum after 72 hours and stayed at this level until the end of the experiment (Fig. 3.7).

Although free paracetamol was detected in the shoots in a small concentration, no conjugates could be detected. The presence of metabolism however cannot be excluded. In the first experiment (1 mM) the free paracetamol concentration in shoots is about 30 μg/g FW and the amount of conjugates was near the detection limit. In case of lower paracetamol exposure, where the concentration is about 0.08 μg/g FW (0.3% of the previous concentration), the amount of conjugates could be below the detection limit.

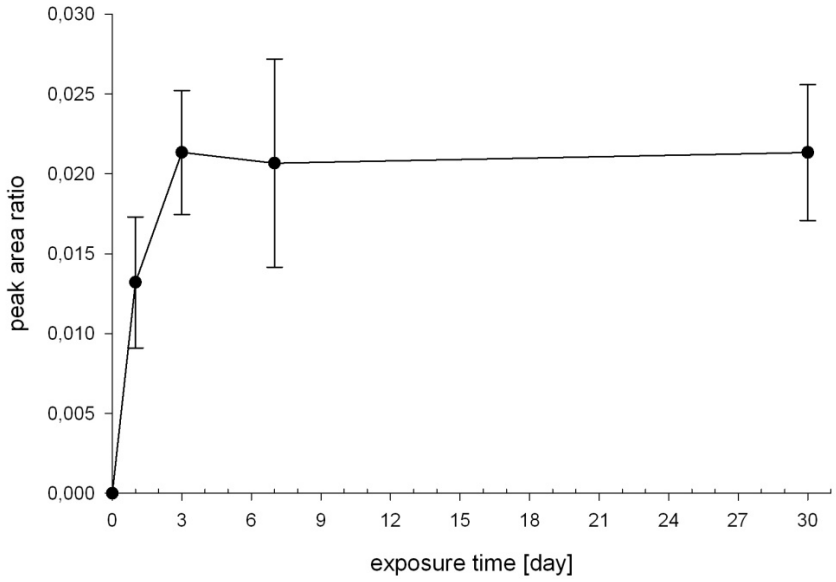


Figure 3.7 Relative amount of paracetamol-GS in *Typha* root tissues after 1 mg/L pharmaceutical exposure.

3.1.4 Effect of paracetamol on the Glycosyltransferase activity in *Typha*

Glycosyltransferases play an important role in the detoxification of agrochemicals and xenobiotics, but also in the plant cellular homeostasis, because these enzymes catalyze the conjugation of many endogenous substrates like plant hormones or plant secondary metabolites. To determine enzyme activity changes induced by 1 mg/L paracetamol exposure, different substrates were used in the assay: a model xenobiotic compound 2,3,4-trichlorphenol (TCP) and the plant-derived flavonoids quercetin and kaempferol. Table 3.2 represents the activity of GT in *Typha* root and shoot tissues for these substrates.

Using the xenobiotic TCP as substrate, no enzyme activity was detected in roots. In the shoots fast GT activity increase was observed; after 24 hours maximum increase in treated plants (up to 662% of control) was followed by continuous decrease over the exposure time. In spite of this activity depletion, treated plants still have elevated GT activity in their shoots (175% of control).

Measurements with the two endogenous substrates quercetin and kaempferol showed an activation of GT enzyme both in root and leaf tissues after exposure to paracetamol. The GT-quercetin activity in roots increased to 138% of control after 24 hours of exposure. It reached a maximum three days after treatment (220% of control) and showed elevated activity till the end of the experiment. Contrary to this, using kaempferol as substrate no increase was observed in the roots; even lower activities were measured compared to control.

In plant leaves a fast and significant activity increase was observed during exposure. In case of quercetin the enzyme activity was increased to 528% of control after 24 hours and showed continuously elevating activity. GT-kaempferol activity reached its maximum already after 24 hours of exposure at 684% of control followed by decreasing activity during the first week. However, the enzyme activity, at 471-525% of control, was still elevated. After 30 days the GT activity was decreased, but still 292% of control.

Paracetamol treatment leads to GT enzyme activation in *Typha*, both in root and shoot tissues. However no measurable conjugation product with glucose was determined in the plants. The results refer to detoxification via glycosylation in very small amounts, but the main phase II process during the low concentration paracetamol exposure seems to be the conjugation with glutathione. The LC-MS measurements confirm this assumption, since the paracetamol-glucopyranoside was not clearly detected in the plants. Nevertheless trace amounts of the conjugate were found in a few samples, but data were at the detection limit.

Table 3.2 Glycosyltransferase activity in *Typha* control and 1 mg/L paracetamol treated plants. Changes in enzyme activity are also represent as control %.

GT activity [pkat/mg protein]						
	Roots			Shoots		
Time [day]	TCP			TCP		
	control	paracetamol	control%	control	paracetamol	control%
1	n.d.	n.d.	n.d.	0.8±0.03	5.3±0.1	662%
3	n.d.	n.d.	n.d.	1.01±0.02	3.4±0.01	339%
7	n.d.	n.d.	n.d.	0.87±0.03	2.1±0.01	239%
30	n.d.	n.d.	n.d.	1.2±0.02	2.2±0.01	175%

	quercetin			quercetin		
	control	paracetamol	control%	control	paracetamol	control%
1	1.92±2E-04	2.65±2E-04	138%	1.57±3E-03	8.3±1E-03	528%
3	1.4±4E-04	3.1±4E-04	220%	1.53±5E-03	5.5±7E-04	756%
7	0.97±6E-05	2.1±4E-04	217%	2.2±1E-03	8.8±5E-04	647%
30	1.33±4E-04	2.7±2E-06	202%	2.01±1E-03	9.8±5E-03	328%

	kaempferol			kaempferol		
	control	paracetamol	control%	control	paracetamol	control%
1	0.85±6E-04	0.73±2E-04	86%	1.1±3E-03	7.4±3E-04	684%
3	1.52±1E-04	2.16±5E-04	142%	1.65±4E-04	3.6±6E-04	471%
7	1.46±2E-05	0.86±3E-04	59%	1.6±1E-03	5.4±5E-04	525%
30	1.33±3E-04	0.61±4E-05	46%	1.6±8E-04	6.9±3E-03	292%

3.1.5 Effect of paracetamol on the Glutathione S-transferase activity in *Typha*

1mM paracetamol exposure

Glutathione S-transferase activity was measured in cytosolic extract of *Typha* plants exposed to 1 mM paracetamol using CDNB and NBOC as substrate.

Enhanced GST-CDNB activity was observed in the root tissues after 24 and 72 hours paracetamol treatment, however without statistical significance (Fig. 3.8 a). In the shoot tissues there was no significant change in the activity of the enzyme, although a slight increase after 72 hours exposure was measured (Fig. 3.8 b).

In contrast GST-NBOC activity showed no significant difference in the root tissues of the treated plants compared to the control group. However GST activity in the shoot increased moderately after 24 and 72 hours and after one week a significant increase of activity (246% of control) was observed (Fig. 3.8 c-d).

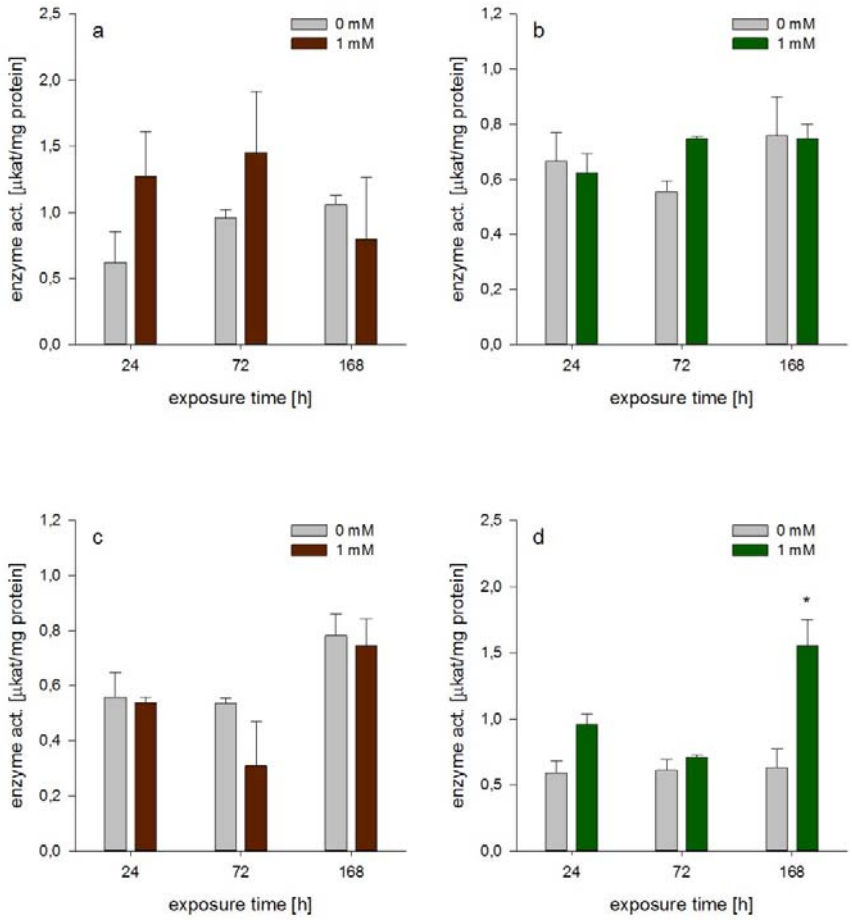


Figure 3.8 GST-CDNB activity in *Typha* roots (a) and shoots tissues (b) and GST-NBOC activity in roots (c) and shoots (d) under 1 mM paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. *p<0.05 vs. control.

1 mg/L paracetamol exposure

Exposed to 1 mg/L paracetamol GST activities showed marked differences to the high concentration. At first a significant decrease of GST-CDNB activity in the roots took place after 24 and 72 hours, followed by increasing activity after one week. At the end of the experiment, i.e. after 30 days, the activity increased to 160% of control (Fig. 3.9 a). In the plant shoots a fast and significant activity increase was observed during the first 3 days exposure time (Fig. 3.9 b).

GST activity using NBOC as substrate increased to 235% of control and showed elevated activity after 3 and 7 days, similar to the shoot, where the enzyme activity tends to increase as well (Fig. 3.9 c-d).

Enzyme activities were also measured using fluorodifen, a herbicide described as a specific substrate for Tau class GST enzymes (Fig. 3.10). During the experiment GST activity increased markedly in the plant roots (200% and 192% of control at 24 and 72 hours). After 3 days activity slightly decreased, but nonetheless was significantly higher than in the control (154% and 121% of control). On the other hand changes in the plant shoots were not significant and tend to decrease compared to the control group.

Since many metabolic steps in phase I reactions are membrane bound processes like the activation of xenobiotics by microsomal P450 enzymes, a specific role of microsomal GSTs in the detoxification could be suggested. Glutathione S-transferase activity was measured in microsomal extracts of *Typha* plants exposed to 1 mg/L paracetamol using CDNB, NBOC and fluorodifen as substrates.

Using CDNB, transient enzyme activity increases were observed both in root and in shoot tissues (Fig. 3.11 a-b). Fast enzyme activation in the plant shoots was observed using NBOC as substrate and reached its maximum level after 3 days, whilst in the roots the enzyme activity was decreased (Fig. 3.11 c-d). Significant enzyme activation in the roots was determined when fluorodifen was used as a substrate after 24 hours of exposure followed by a continuous activity decrease over time compared to the control level. In the shoots only a slight increase after 1 and 3 days of exposure was observed (Fig. 3.11 e-f).

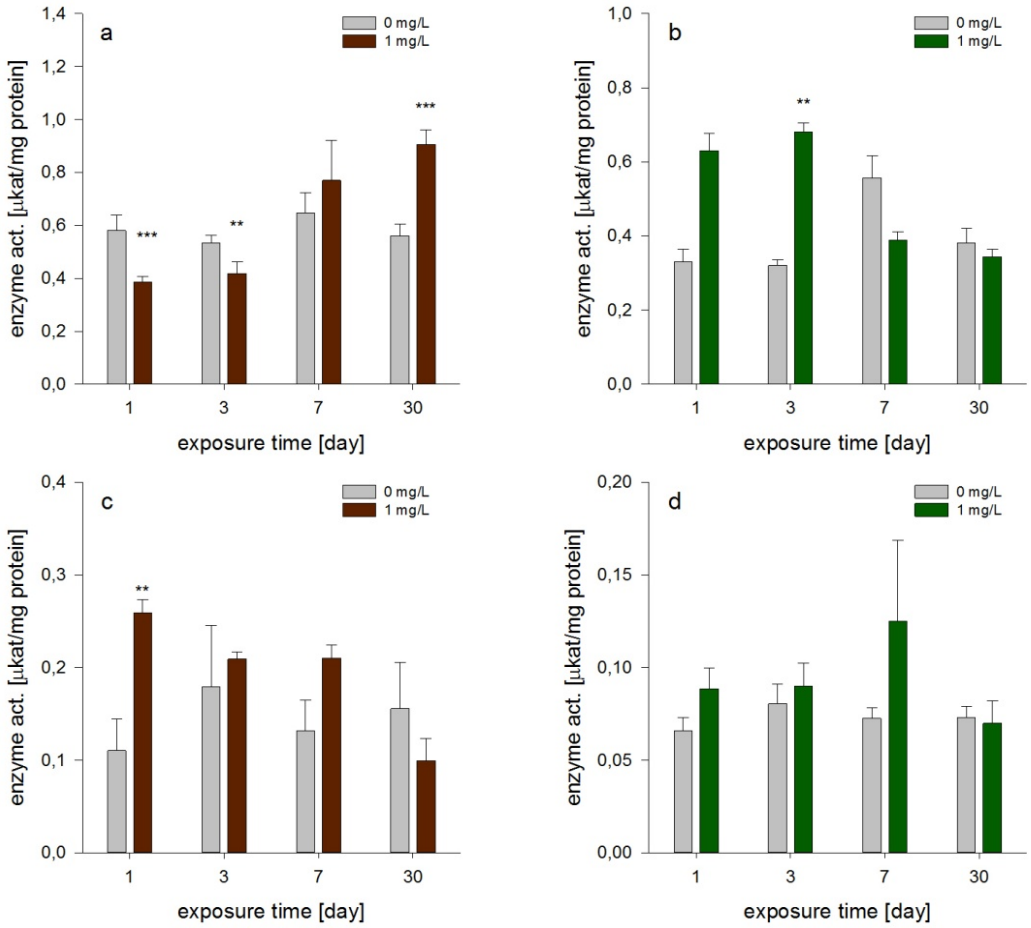


Figure 3.9 GST-CDNB activity in *Typha* roots and shoots (a-b) and GST-NBOC activity in roots and shoots (c-d) under 1 mg/L paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. **p<0.01, ***p<0.001 vs. control.

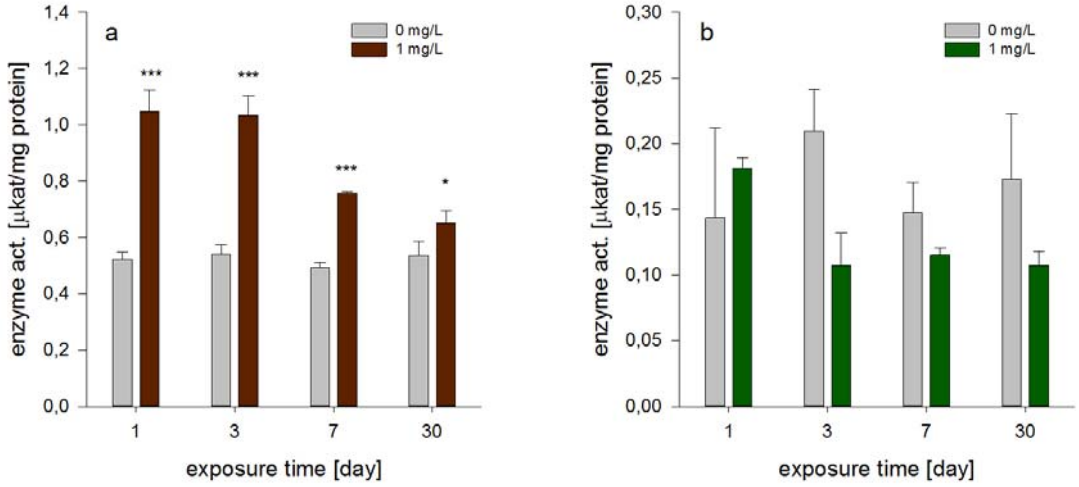


Figure 3.10 GST-fluorodifen activity in *Typha* roots (a) and shoots (b) under 1 mg/L paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. *p< 0.05, **p< 0.01, ***p<0.001 vs. control.

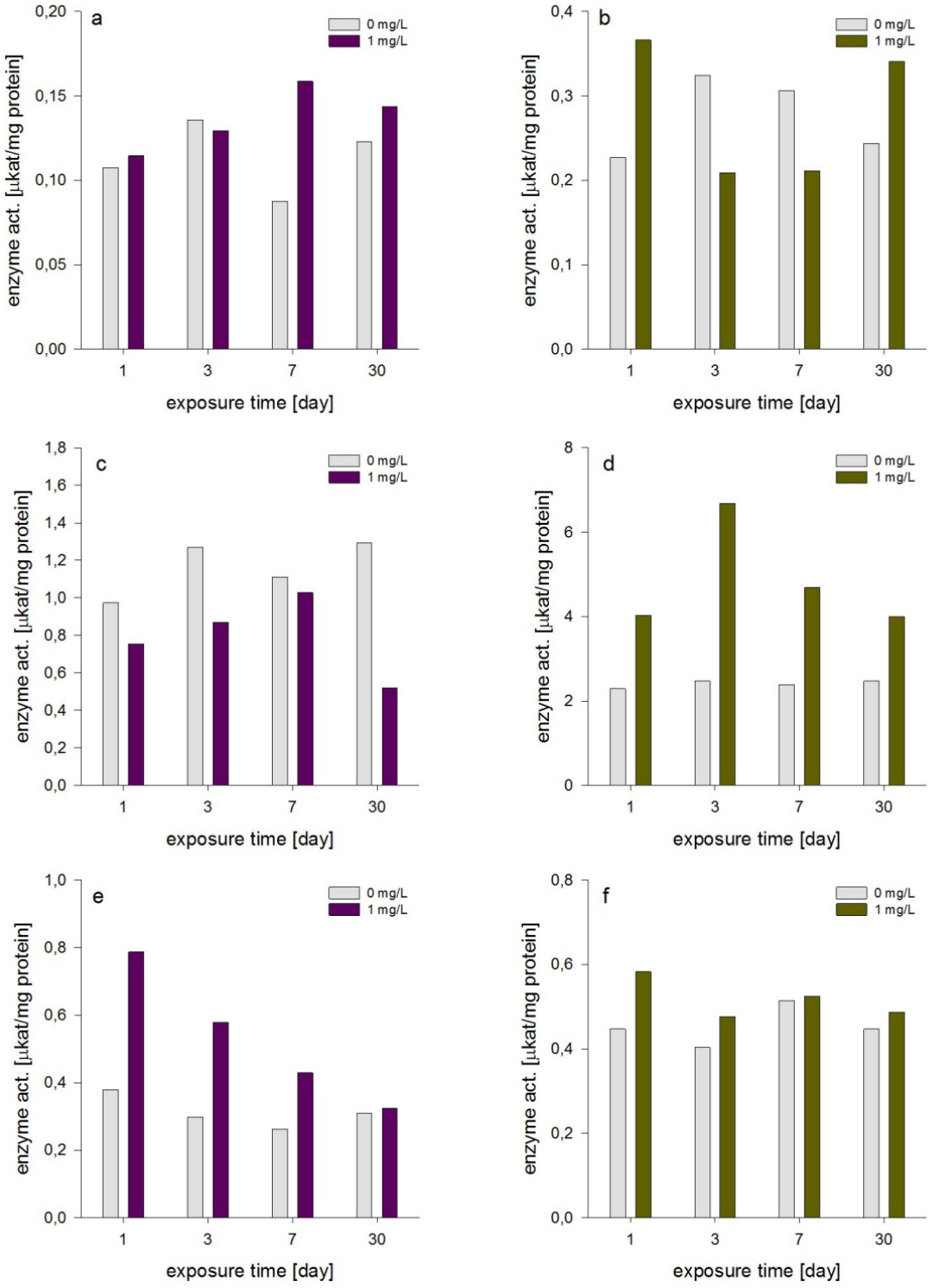


Figure 3.11 Microsomal GST-CDNB activity in *Typha* roots and shoots (a-b), GST-NBOC activity in roots and shoots (c-d) and GST-fluorodifen activity in roots and shoots (e-f) under 1 mg/L paracetamol exposure. Fresh material of three biological replicates was pooled during the extraction to reach the required fresh weight.

3.1.6 Oxidative stress and enzymatic defense responses in *Typha*

Reactive oxygen species (ROS) production during various metabolic activities are not inevitable. Natural processes controlled by enzymatic and non enzymatic defense systems maintain the redox balance and keep the plant cells functioning. Various biotic and abiotic stresses lead to the overproduction of ROS, which result in oxidative stress in plants. Without effective defense responses this leads to fatal cell damages (Noctor et al. 2012).

Ascorbate peroxidase and Catalase play a crucial role in H_2O_2 scavenging. APOX is involved in the Halliwell-Asada pathway and utilizes ascorbic acid as an electron donor to decompose hydrogen peroxide in the micro molar range to water. CAT is important in the removal of H_2O_2 generated in peroxisomes in the mM range concentration, but less efficient in removing low concentrations.

1 mM paracetamol exposure

In roots, exposure to 1 mM paracetamol APOX showed increasing activity that reached a maximum one week after treatment, while in shoot slightly enhanced activity was measured after 24 and 72 hours (Fig. 3.12 a-b). There was no significant change in the activity of CAT (Fig. 3.12 c-d).

Glutathione reductase (GR) catalyzes the NADPH dependent regeneration of GSSG back to GSH in the Halliwell-Asada pathway, so playing an important role in the ascorbate-glutathione cycle. GR activities 24, 72 and 168 hours after the treatment with 1 mM paracetamol were decreased to 58%, 45% and 39% of the control in root (Fig. 3.13 a). On the other hand, enzyme activity in shoot was increased and reached its maximum after 72 hours at 353% of the control (Fig. 3.13 b).

Peroxidases (POX) are also involved in ROS elimination due to the reduction of H_2O_2 using various phenolic compounds as electron donors and play a role in the degradation of toxic molecules. Whereas POX activity changes in roots were not significant, in shoot the enzyme activity increased to 585% and 485% of control after 24 and 72 hr (Fig. 3.13 c-d). Even after one week significantly increased activity (225% of control) was measured.

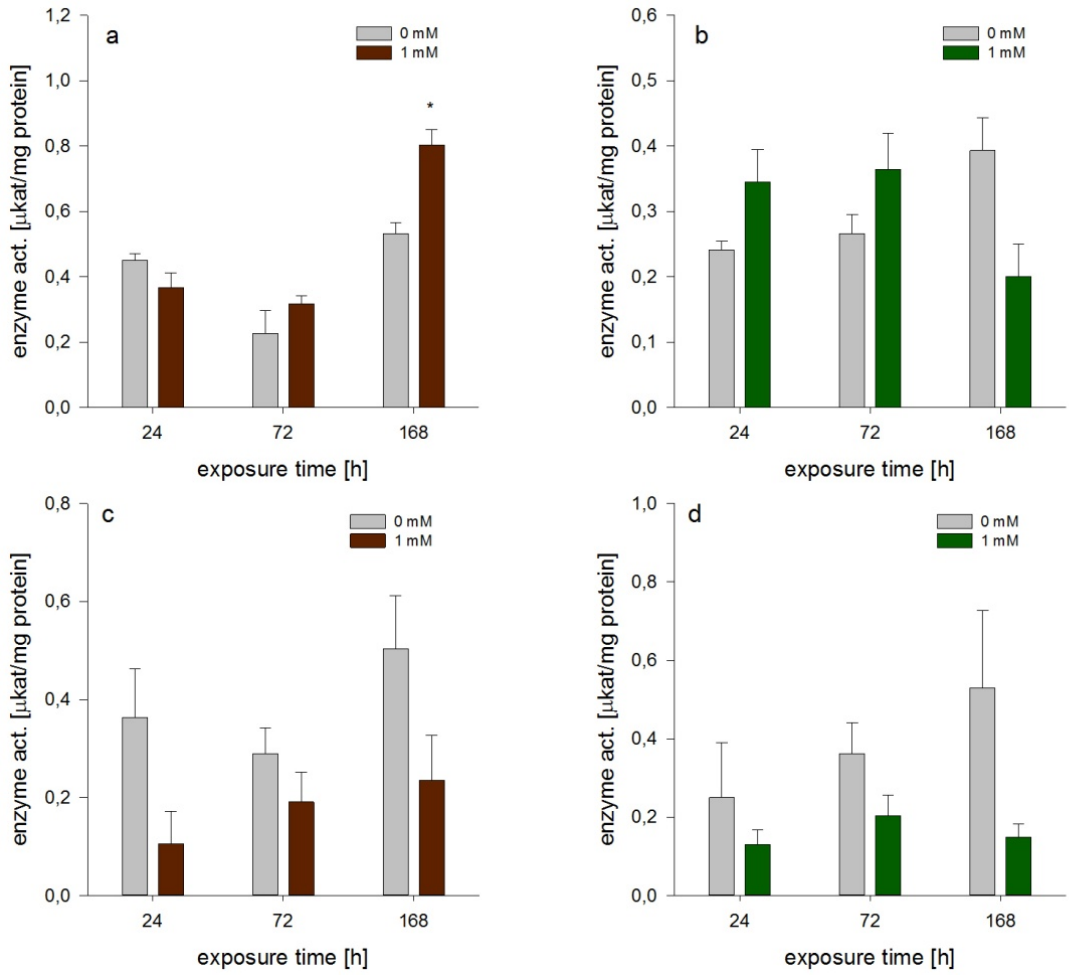


Figure 3.12 Specific enzyme activities in *Typha* under 1 mM paracetamol exposure: APOX in roots and shoots (a-b), CAT in roots and shoots (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05 vs. control.

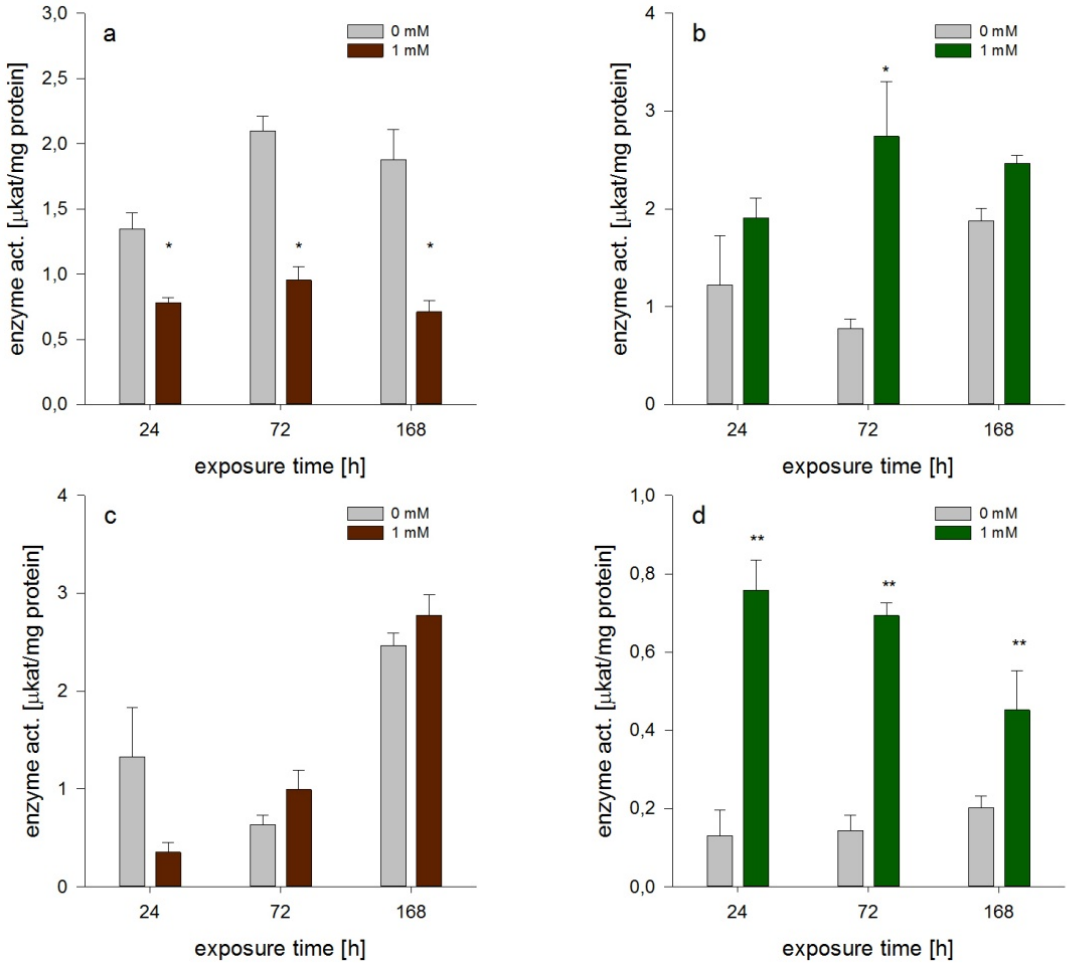


Figure 3.13 Specific enzyme activities in *Typha* under 1 mM paracetamol exposure: GR in roots and shoots (a-b), POX in roots and shoots (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01 vs. control.

1 mg/L paracetamol exposure

In case of treatment with 1 mg/L paracetamol a clear activation of the APOX enzyme was observed during the whole experiment both in root and shoot tissues. In roots the activity increase was moderate, at 125% of control after one day exposure, followed by further increase up to 170% of control after three days and kept its activity level till the end of the experiment (Fig. 3.14 a). APOX in the shoots showed a much stronger effect with a 4-fold increased activity after one day of exposure. However after that the enzyme activity decreased and showed a similarly moderate activation (about 130% of control) as in plant roots till the end of the paracetamol treatment (Fig. 3.14 b).

The Glutathione reductase activity was also increased after the pharmaceutical exposure. The enzyme activity in treated roots was about 130% of control level during the

first week of exposure, but decreased back to the control level after 30 days (Fig. 3.15 a). Similarly, slightly increased GR activity was observed in shoots over the exposure time, although the result of three biological replications showed no significance (Fig. 3.15 b).

Remarkably, CAT activity in the samples was always at below detection limits, which may refer to moderate H_2O_2 production in the plants under the low-concentration paracetamol exposure and so indicates a lower oxidative stress status.

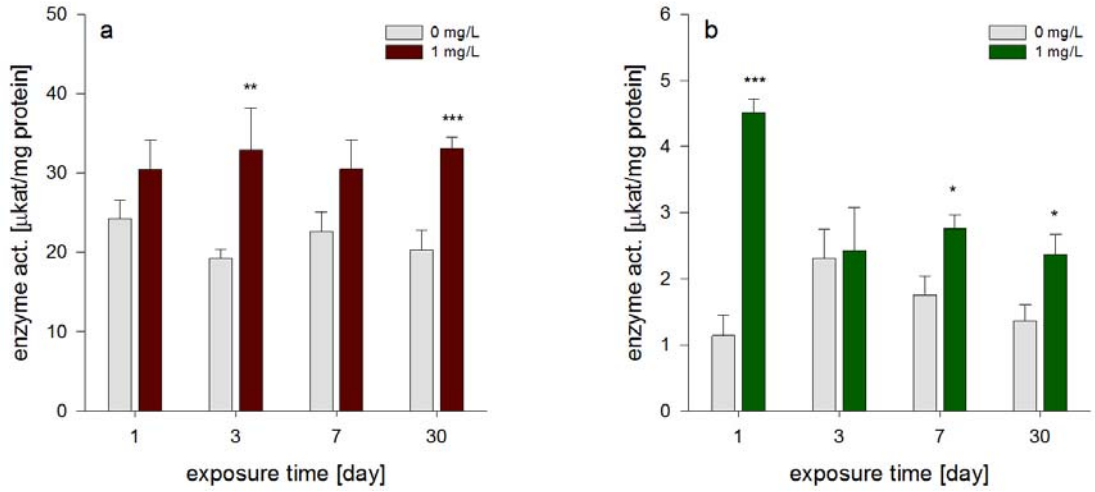


Figure 3.14 Specific enzyme activities in *Typha* under 1 mg/L paracetamol exposure: APOX in roots (a) and shoots (b). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

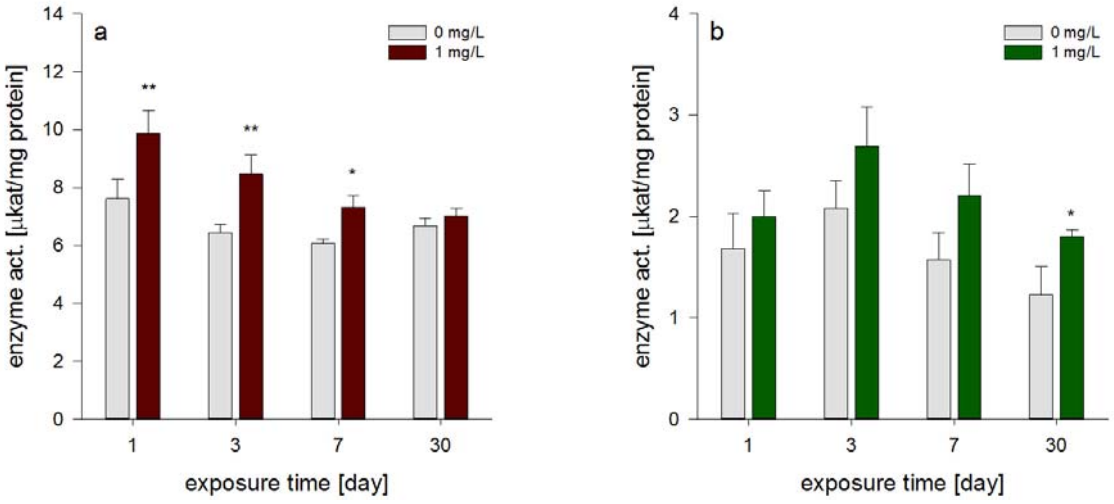


Figure 3.15 Specific enzyme activities in *Typha* under 1 mg/L paracetamol exposure: GR in roots (a) and shoots (b). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01 vs. control.

Cytosolic POX activity

After exposure to 1mg/L paracetamol POX enzyme activity was measured in cytosolic and in microsomal extracts of *Typha* root and shoot tissues. In the cytosolic fraction of root tissues POX activity showed strong activation; increased to 500% of control already after one day of exposure and kept this level till the end of the experiment (Fig. 3.16 a). This strong activation in shoots was not observed. During the first week no significant change was detected in the treated plants. Only after 30 days of exposure was the POX activity increased to 170% of control (Fig. 3.16 b).

Microsomal POX activity

The enzyme activity changes in roots were not as heavily affected as the cytosolic enzyme activity; no significant change was detected during the first three days after treatment. After that a moderate enzyme activity increase (to 146% of control) was observed after seven days of exposure, which showed further decrease at the end of the 30 days after treatment (Fig. 3.16 c). Contrary to this, POX showed significant activation in shoots, where increased enzyme activity was observed up to 300% of control after three days of exposure. Yet after 30 days the activation effect was recovered and enzyme activity decreased to the control level (Fig. 3.16 d).

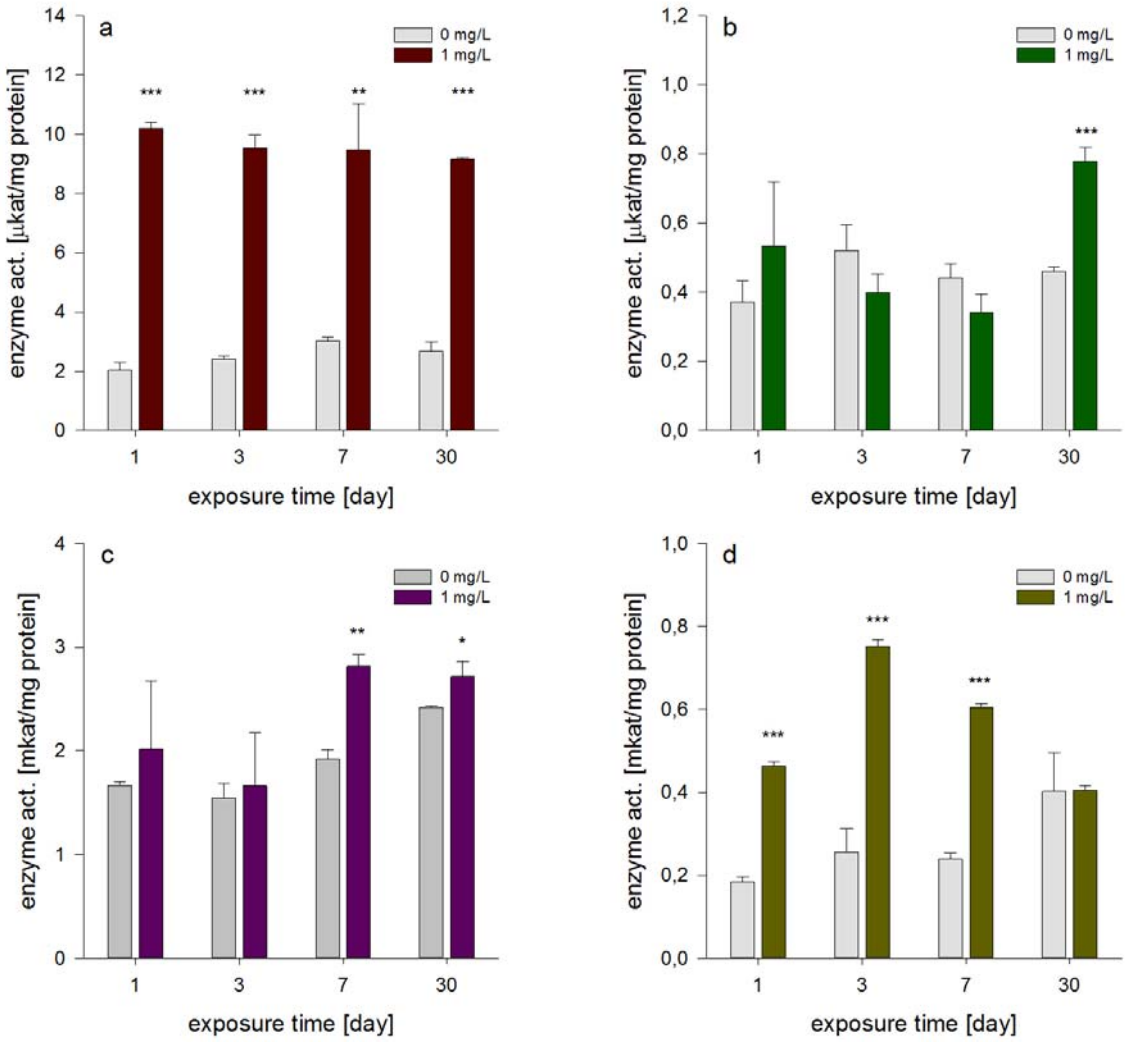


Figure 3.16 Specific POX enzyme activities in *Typha* under 1 mg/L paracetamol exposure: cytosolic enzyme activity in roots and shoots (a-b), and microsomal enzyme activity in roots and shoots (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

3.2 **Paracetamol uptake and detoxification in *Brassica juncea***

3.2.1 Uptake and translocation of paracetamol

1 mM paracetamol exposure

Paracetamol concentration was determined in the incubation medium in the absence and presence of plants under 1 mM paracetamol exposure. Uptake by the plants is significant in the first 72 hours, but thereafter a subsequent decrease of paracetamol in the medium in both cases was observed, suggesting the presence of a plant independent abiotic process that decreased the available amount of paracetamol (Fig. 3.17). However, while the availability of paracetamol is strongly decreased, the uptake by plants was still significant (insert in Fig. 3.17).

LC-MS analysis of root and leaf samples showed that a considerable amount of paracetamol was taken up from the growth medium. After 24 hours paracetamol was detected in the highest amount in root (0.42 mg/g FW) and also in the leaf tissues (0.26 mg/g FW). After one week treatment a strong decrease of paracetamol was observed in both tissues (Fig. 3.18 a).

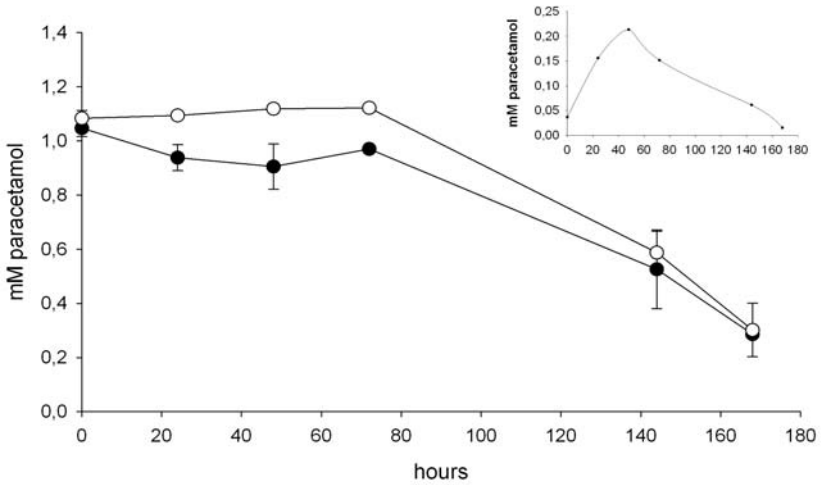


Figure 3.17 Paracetamol concentration in the incubation medium in the absence (○) and the presence (●) of plants. The insert depicts the uptake of paracetamol in plants as difference between the two curves. Error bars represent ± S.D. of experimental triplicates.

1 mg/L paracetamol exposure

Plants exposed to 1 mg/L of pharmaceutical showed also fast uptake in the root tissues: after 24 hours 0.13 µg/g FW and after 72 hours 0.14 µg/g FW paracetamol was measured (Fig. 3.18 b). The transport to the upper part was moderate; it reached its maximum already after 24 hours, when 15% of paracetamol was detected in leaves.

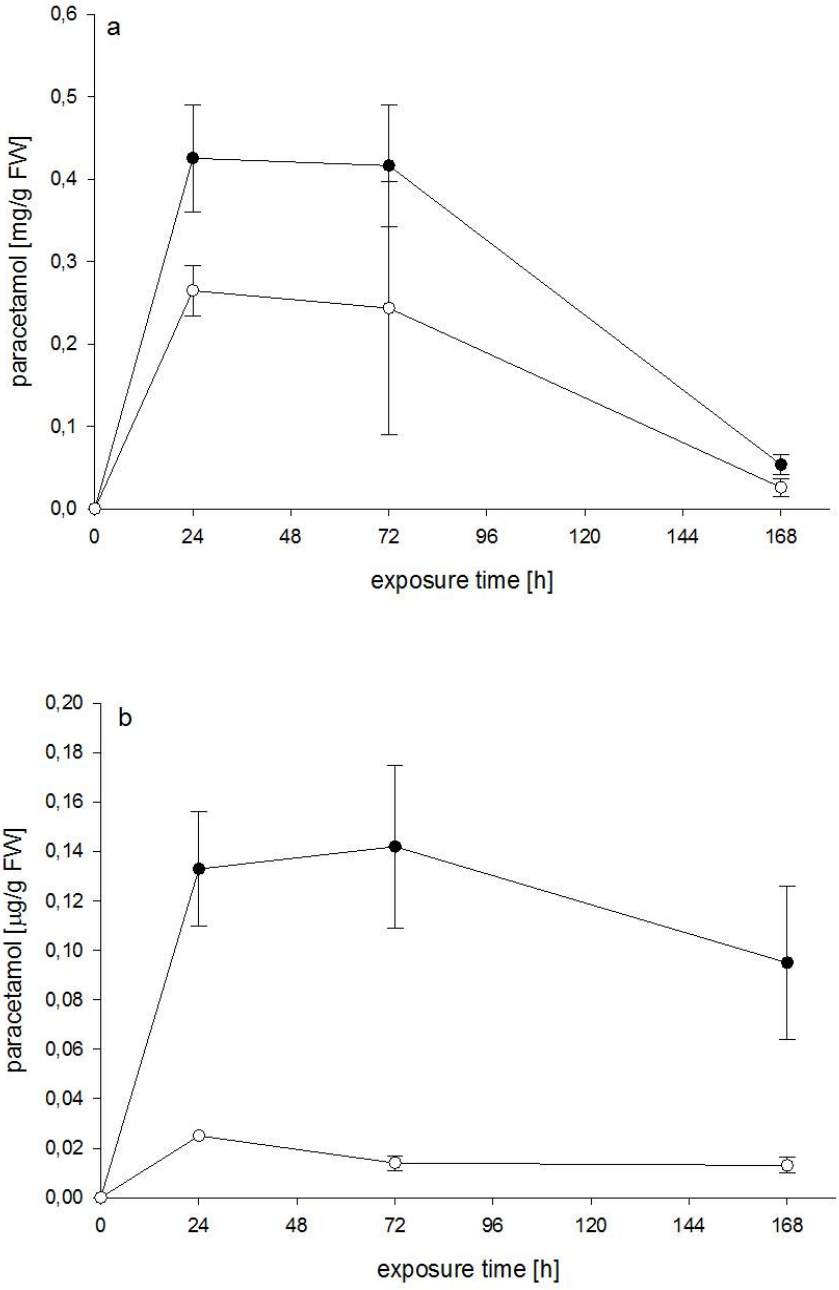


Figure 3.18 Paracetamol uptake and transport in *Brassica* root (●) and leaf (○) tissues. a: 1 mM pharmaceutical exposure, b: 1 mg/L pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

3.2.2 Paracetamol metabolites in *Brassica juncea*

LC-MS/MS analysis allowed the detection of paracetamol-glutathionyl and paracetamol-glycoside conjugates as in *Typha* plants. Additionally in *Brassica* plants paracetamol-cysteine conjugate was identified as a third metabolite. The cysteine conjugate is formed from the paracetamol-GS conjugate due to the loss of glycine and γ-glutamin (Fig. 3.19). The product was detected due to the molar mass transition of m/z 271 → 182 as described by Mutlib et al. (2000).

1 mM paracetamol exposure

After exposure to 1 mM paracetamol all the three conjugates were identified in root and leaf tissues. After 24 hours treatment the relative amount of the glutathione conjugate was approximately tenfold higher than the glycoside in the plant roots. The cysteine residue was present in the lowest amount. Over time paracetamol-GS showed a continuous decrease, while the amount of glucopyranoside increased. After one week treatment the two conjugates were present approximately in the same amount. The relative amount of paracetamol-cysteine conjugate was quite low; about 15% of the paracetamol metabolites (Fig. 3.20 a).

In the leaf samples the amount of conjugated paracetamol after 24 hours was low, however after 72 hours a significant increase of conjugation products was observed. While the glutathione conjugate was the main metabolite in root tissue, the amount of paracetamol-glucopyranoside in leaves was fivefold higher than the glutathione conjugate and showed no further decrease one week after treatment. The paracetamol-GS conjugate was degraded to the cysteine residue over the treatment time (Fig. 3.20 b).

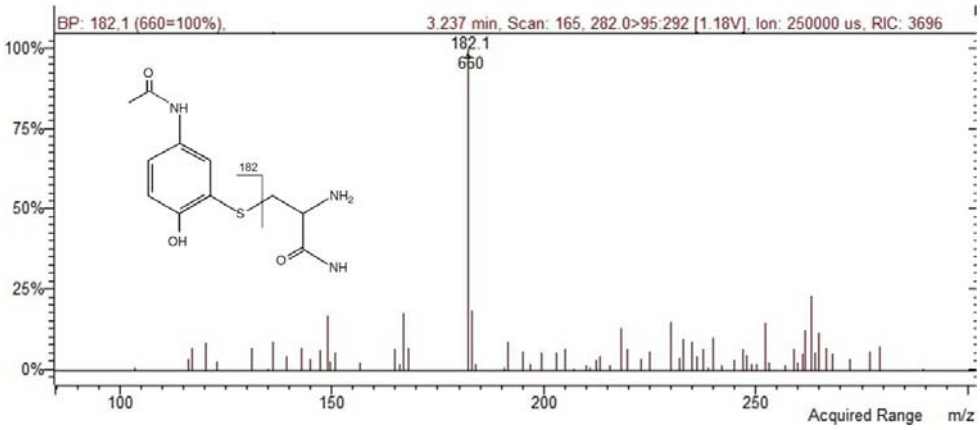


Figure 3.19 MS/MS spectra of cysteinyl paracetamol $(M+H)^+$ *m/z* 271 in *Brassica juncea* root tissues using positive ionization mode.

1 mg/L paracetamol exposure

Glutathionyl and cysteine conjugates were detected in plants exposed to 1mg/L paracetamol both in root and leaf tissues. After 24 and 72 hours the relative amount of paracetamol-GS in root was tenfold higher than the cysteine conjugate. After 168 hours the amount of the conjugate was strongly decreased, however no further increase of the cysteine-residue was detected (Fig. 3.20 c).

In the leaves paracetamol-GS was first detected after 72 and 168 hours, while the cysteine conjugate was already present after 24 hours; approximately in the same amount as in root tissues. At the end of the experiment 62% of the paracetamol metabolites were identified as glutathione conjugates and 38% as cysteine conjugates (Fig. 3.20 d).

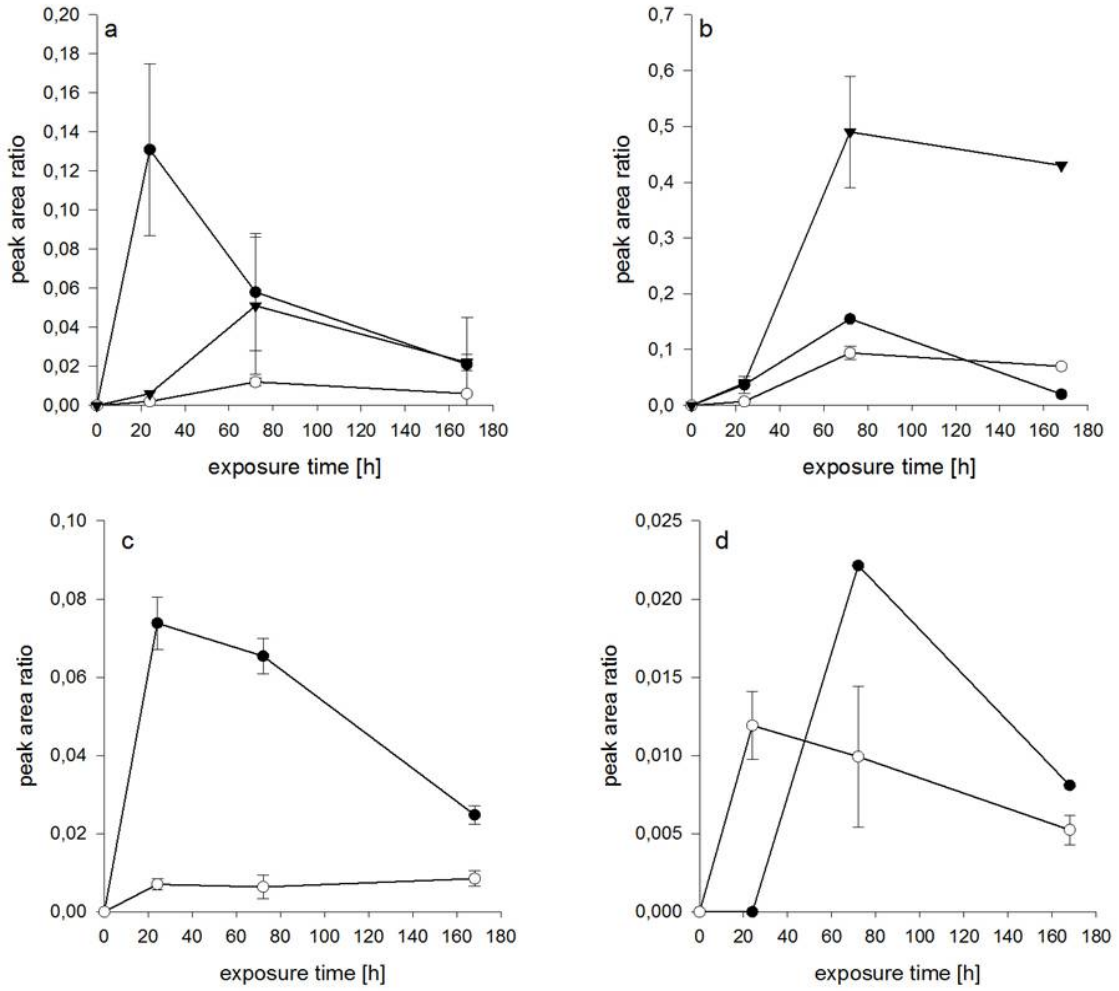


Figure 3.20 Relative amounts of paracetamol-GS (●), paracetamol-cysteine (○) and paracetamol-glucopyranoside (▼) conjugates in *Brassica* roots and leaves (a-b) after 1 mM pharmaceutical exposure, and in roots and leaves (c-d) after 1mg/L exposure. Error bars represent ± S.D. of experimental triplicates.

3.2.3 Effect of paracetamol on the Glycosyltransferase activity in *Brassica*

After exposure to 1mg/L paracetamol GT enzyme activity was measured in cytosolic extracts of *Brassica* root and shoot tissues using three substrates; the model xenobiotic compound 2,3,5-TCP and the flavonoids quercetine and kaempferol as endogenous enzyme substrates. Table 3.3 represents the activity changes of GT in *Brassica* root and leaf tissues.

In contrast to the results of the *Typha* experiment no induction in the GT activity was determined, neither in root nor in leaf tissues. In roots slightly decreased enzyme activity was observed; 48% of control after seven days of pharmaceutical exposure. The

effect of paracetamol exposure was markedly reduced enzyme activity in leaf tissues. At 24 hours after the treatment the GT activity was decreased to 20% of control with TCP, to 28% of control with quercetin and to 34% of control with kaempferol as respective substrate. In spite of further enzyme activity increase (after 72 and 168 hours of exposure) the plants were not able to recover their GT activity in the shoot tissues.

Table 3.3 Specific Glycosyltransferase activity in *Brassica* control and 1 mg/L paracetamol treated plants. Changes in enzyme activity are also represented as % of control.

GT activity [pkat/mg protein]						
	Roots			Leaves		
Time	TCP			TCP		
	control	paracetamol	control %	control	paracetamol	control %
24 hr	3.4 ± 0.02	2.2 ± 0.69	66.2 %	9.07 ± 1.5	1.85 ± 0.0	20.4 %
72 hr	4.47 ± 1.7	1.89 ± 0.23	42.3 %	8.34 ± 0.2	2.3 ± 0.46	27.7 %
168 hr	3.76 ± 0.71	1.8 ± 0.11	48 %	8.16 ± 0.12	4.45 ± 0.03	54,6 %
	quercetin			quercetin		
	control	paracetamol	control %	control	paracetamol	control %
24 hr	6.96 ± 0.19	4.49 ± 0.6	64.6 %	28.1 ± 1.3	7.99 ± 0.0	28.4 %
72 hr	7.45 ± 1.6	4.32 ± 0.2	58 %	23.54 ± 0.7	9.67 ± 1.3	41.1 %
168 hr	5.44 ± 0.27	5.03 ± 0.07	92.5 %	27.68 ± 1.03	19.43 ± 3.1	70.2 %
	kaempferol			kaempferol		
	Control	paracetamol	Control %	Control	paracetamol	Control %
24 hr	5.75 ± 0.1	4.21 ± 0.5	72.3 %	23.86 ± 1.03	8.08 ± 0.0	33.9 %
72 hr	6.12 ± 1.3	4.22 ± 0.09	69 %	23.49 ± 0.02	9.53 ± 1.01	40.6 %
168 hr	4.54 ± 0.75	4.75 ± 0.06	104.6 %	24.33 ± 0.19	18.03 ± 2.7	74.1 %

3.2.4 Effect of paracetamol on the Glutathione S-transferase activity in *Brassica*

1 mM paracetamol exposure

Cytosolic GST activity was measured in *Brassica juncea* plants exposed to 1 mM paracetamol using CDNB and NBOC as substrate. Both GST-CDNB and GST-NBOC showed decreased activity in plant roots. Using CDNB as substrate the loss of GST enzyme activity was lower, 70% of control after 24 hours of exposure, and reached its minimum (45% of control) 72 hours after treatment (Fig. 3.21 a). In case of NBOC the enzyme activity was 50% of the control in the treated plants after 24 hours and only 20% of the control after 72 and 168 hours exposure (Fig. 3.21 c).

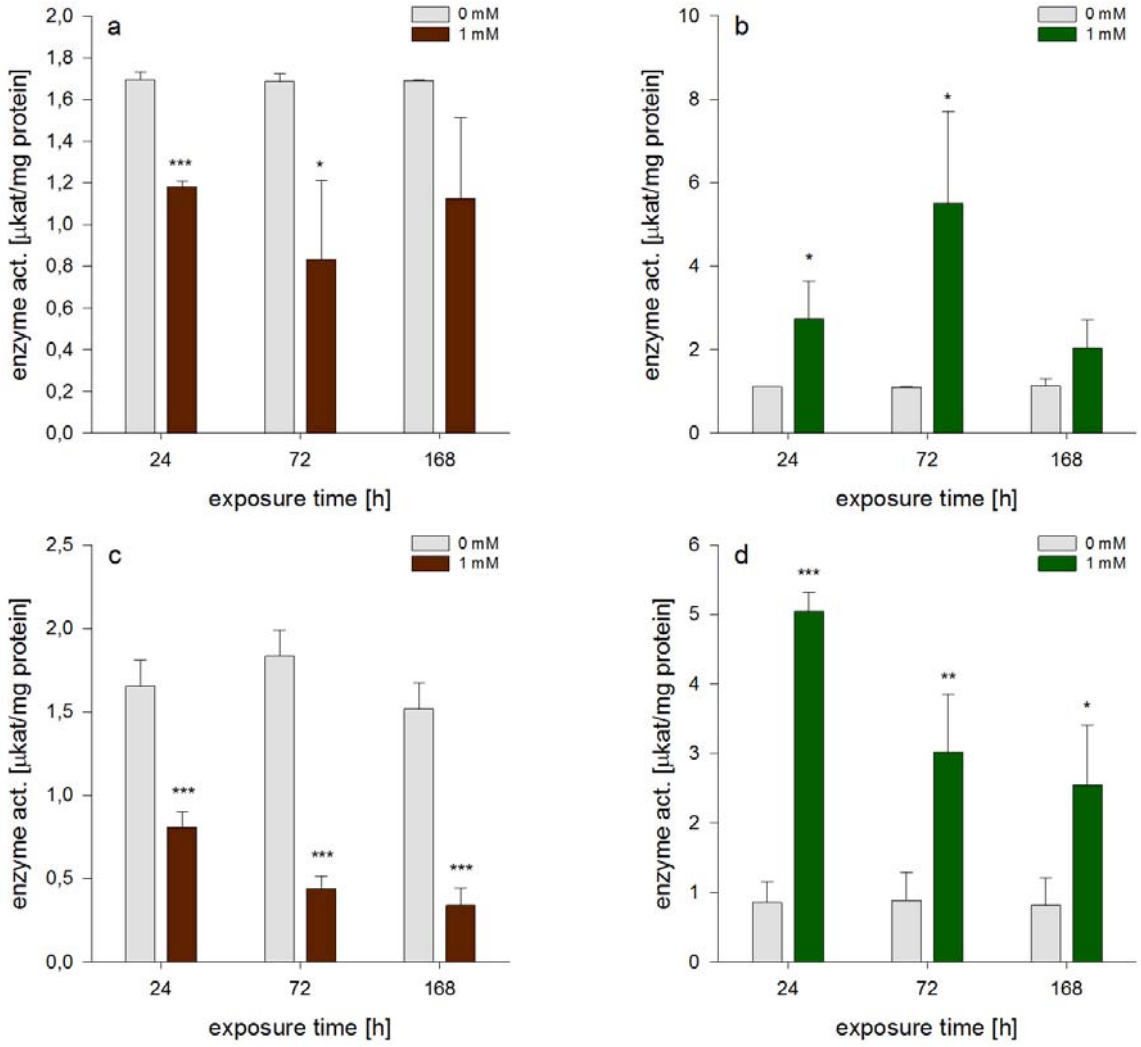


Figure 3.21 GST-CDNB activity in *Brassica* root and leaf tissues (a-b) and GST-NBOC activity in root and leaf tissues (c-d) under 1 mM paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

In contrast strong enzyme activity increase was observed in plant leaves. GST-CDNB activity showed a slower activation effect; the enzyme activity increased to 245% of control after 24 hours of exposure and reached its maximum after 72 hours with a five-fold activity increase compared to control. Although the enzyme activity was decreased after 168 hours of exposure, it was still 180% of control (Fig. 3.21 b). Strong reaction to the pharmaceutical exposure was observed using NBOC as substrate; already after 24 hours of exposure GST activity was increased to 625% of the control. Even after 168 hours significant increase in the activity (312% of control) was still observed (Fig. 3.21 d).

1 mg/L paracetamol exposure

In case of exposure to 1 mg/L paracetamol significant activation of GST was observed in root tissues using CDNB as substrate; about 150% of controls during the whole experiment (Fig. 3.22 a). On the contrary, in leaves at 24 hours after the treatment the enzyme activity was slightly decreased, but no further changes were observed after 72 and 168 hours of exposure (Fig. 3.22 b).

Increased GST activity was observed in root as well as in leaf tissues using NBOC as enzyme substrate. Whereas the increase of the enzyme activity reached its maximum after 168 hours exposure in roots, GST activity in leaves increased to 320% of the control already after 24 hours of paracetamol exposure (Fig. 3.22 c). After 72 hours the enzyme activity was still significantly increased in leaves, but at the end of the experiment the activity in the treated plants decreased to rates close to control level (Fig. 3.22 d).

GST activity was determined in cytosolic extracts of *Brassica* plants exposed to 1 mg/L paracetamol using fluorodifen and para-nitrophenyl acetate (pNpa) as model substrate. In contrast to *Typha* plants, GST activity showed no increased activity in roots using fluorodifen; enzyme activity even decreased after 7 days of exposure (Fig. 3.23 a). In leaf tissues decreased GST-fluorodifen activity was observed as well (Fig. 3.23 b).

Significantly increased GST activity in root tissue was observed during the whole experiment using pNpa as enzyme substrate, up to 160% of control plants after 72 hours of exposure (Fig. 3.23 c). No significant changes in leaf tissues were observed during the exposure time (Fig. 3.23 d).

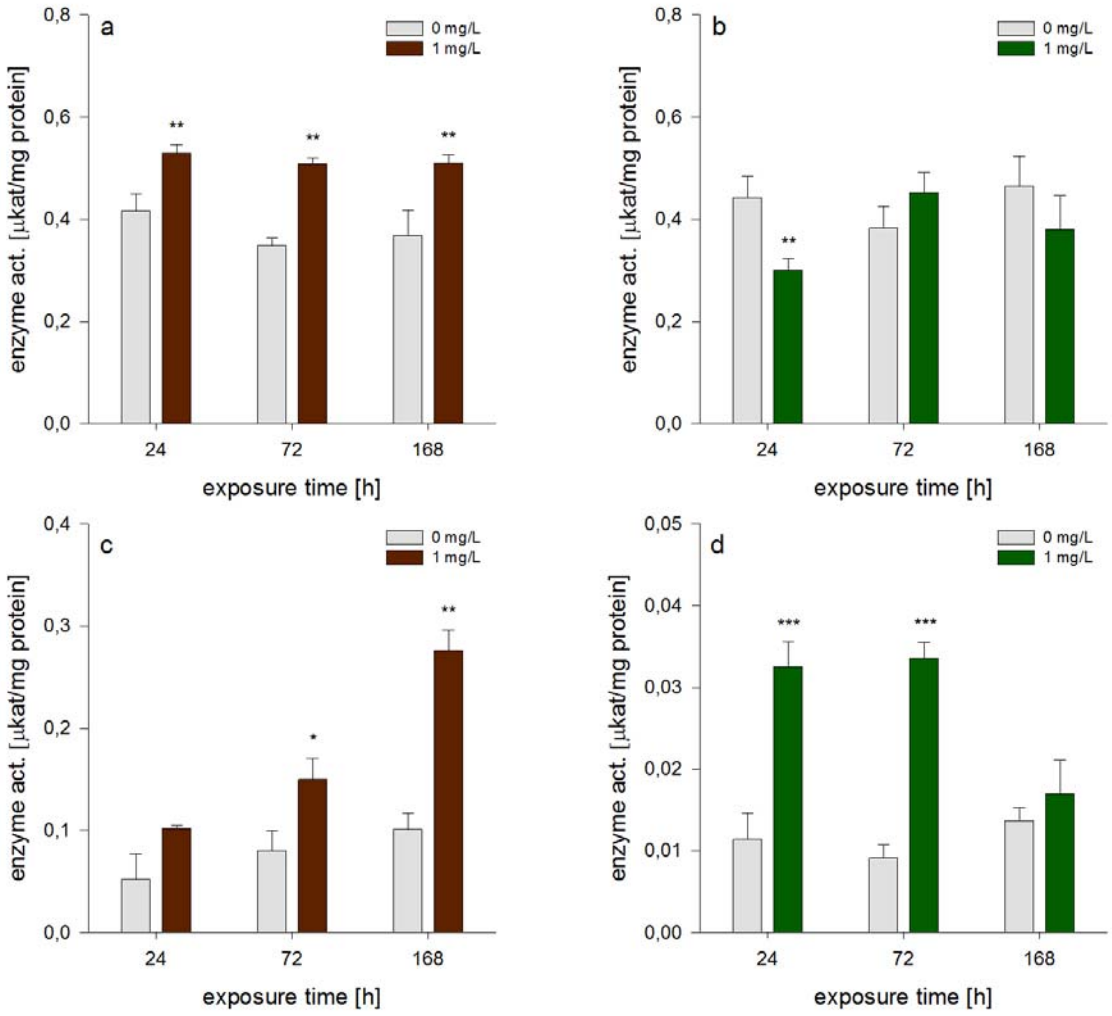


Figure 3.22 GST-CDNB activity in *Brassica* root and leaf tissues (a-b) and GST-NBOC activity in root and leaf tissues (c-d) under 1 mg/L paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

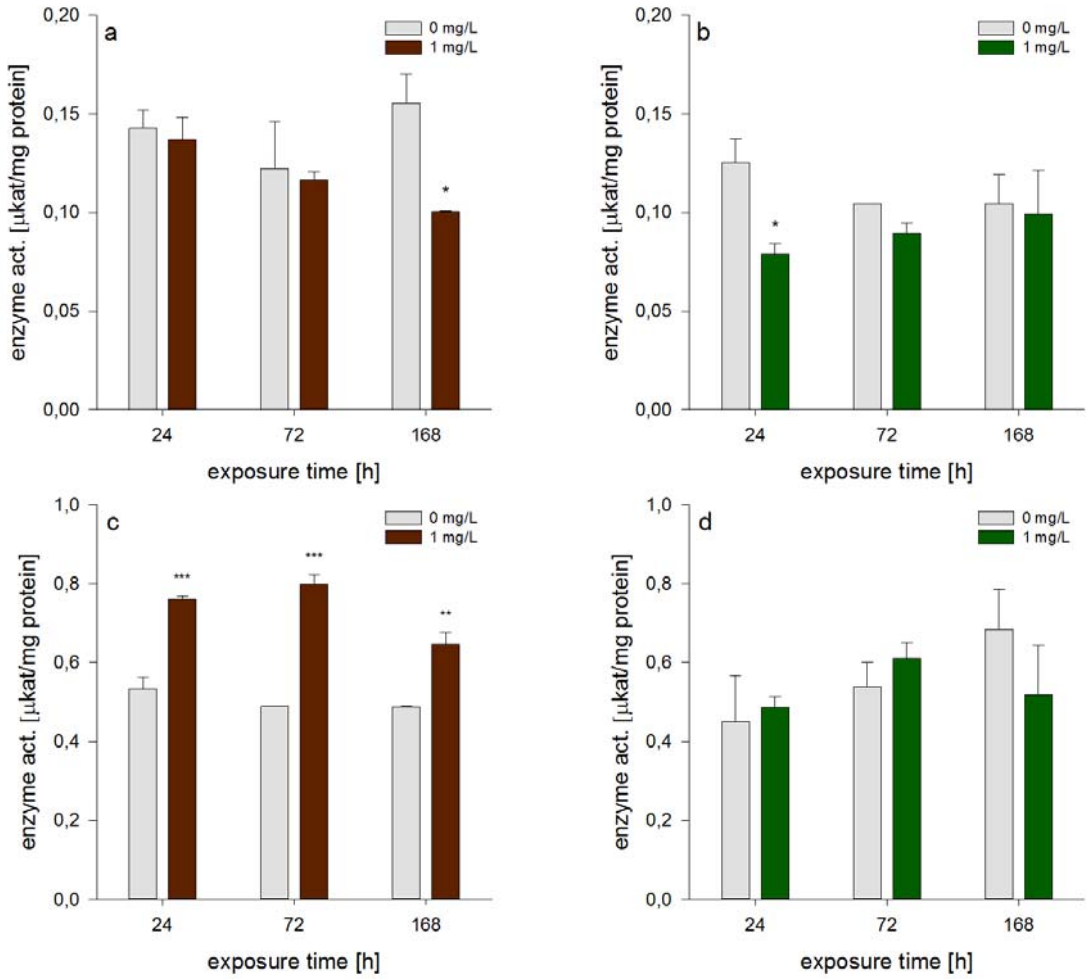


Figure 3.23 GST-fluorodifen activity in *Brassica* root and leaf tissues (a-b) and GST-pNpa activity in root and leaf tissues (c-d) under 1 mg/L paracetamol exposure. Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

Like in *Typha*, Glutathione S-transferase activity was determined in microsomal extracts of *Brassica* plants after 1 mg/L paracetamol exposure. In the assay CDNB, NBOC, fluorodifen and pNpa were used as enzyme substrates. Interestingly, no GST activity was determined in the treated plants, when NBOC was used as a substrate.

Using CDNB, a slight enzyme activity increase was observed in roots 168 hours after treatment (Fig. 3.24 a), whilst in leaf a strong GST activity increase was measured during the whole experiment (Fig. 3.24 b). GST-fluorodifen activity was slightly decreased in root after 24 hours, but showed no further changes compared to control plants (Fig. 3.24 c). In leaf tissues increased enzyme activity was detected (Fig. 3.24 d). Clearly, a positive effect was observed in case of substrate pNpa; increased enzyme activity was measured both in root and in leaf microsomal fraction. However in leaves, this effect ceased after 168 hours (Fig. 3.24 e-f).

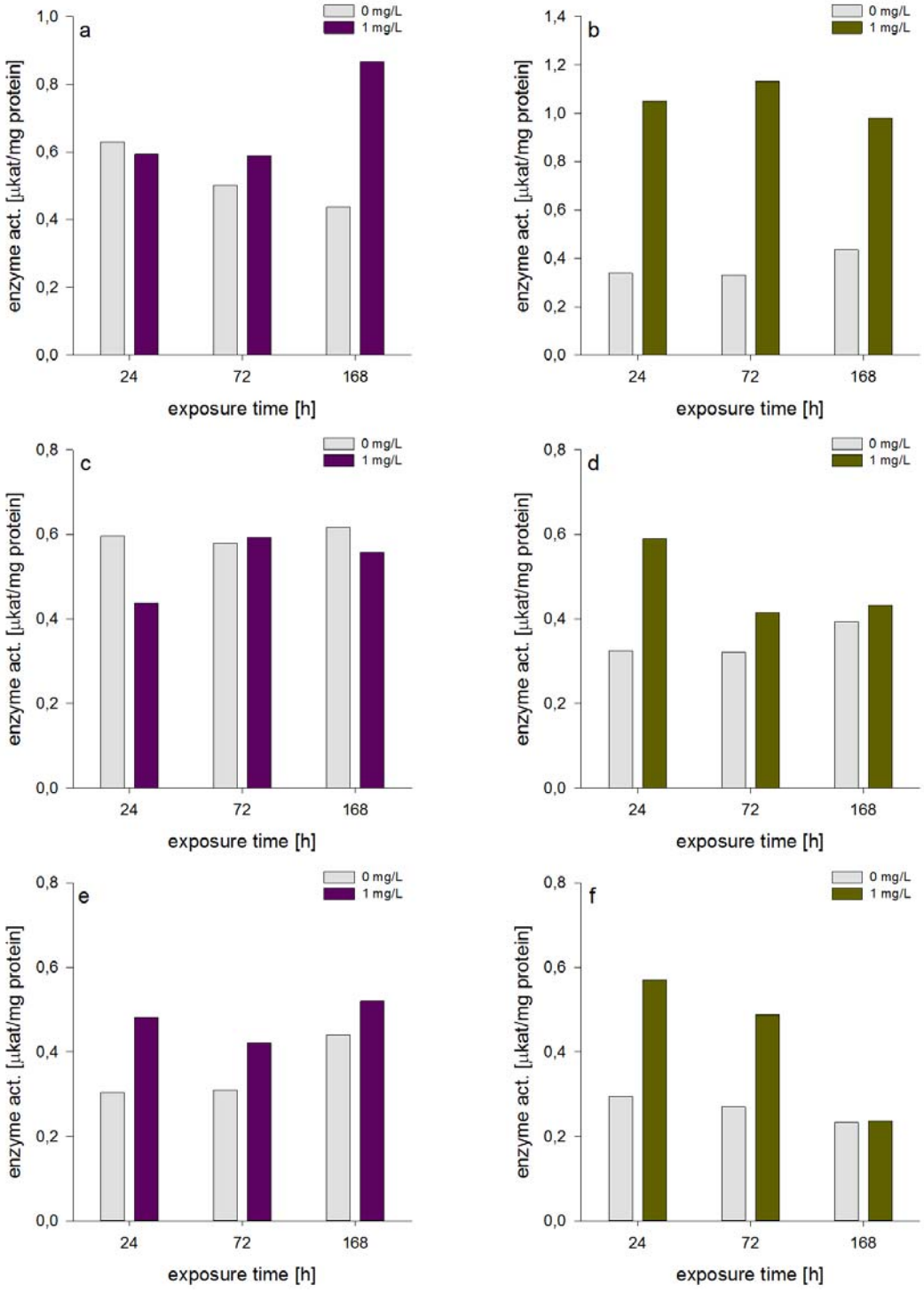


Figure 3.24 Microsomal GST activity in *Brassica*. GST-CDNB activity in roots and leaves (a-b), GST-fluorodifen activity in roots and leaves (c-d) and GST-pNpa activity in roots and leaves (e-f) under 1 mg/L paracetamol exposure. Fresh material of three biological replicates was pooled during the extraction to reach the required fresh weight.

3.2.5 Specific GST gene induction in *Brassica juncea* under 1mg/L paracetamol exposure

Glutathione S-transferase expression in plants may be induced in different stress situations like pathogen attack, drought, high or low temperature, heavy metal or xenobiotic stress. GSTs are thought to play a role in the oxidative stress response mechanisms and in detoxification processes.

The relative gene expression of selected plant specific GSTs was determined in *Brassica* exposed to 1mg/L paracetamol; one member from the GST-Phi group (GST-F6), and three GSTs from the Tau group (GST-U5, GST-U19 and GST-U24).

In *Arabidopsis* plants the up-regulation of GST-F6 has been frequently described. Its expression is induced by biotic and abiotic stress (Lieberherr et al. 2003; Reymond et al. 2000) and can refer to oxidative stress. Here, GST-F6 in *Brassica* was induced in root as well as in leaf tissues. While in roots a continuously increased relative gene expression was observed that reached its maximum after one week of paracetamol treatment (Fig. 3.25 a), the highest induction in leaves was already observed after 24 hours of exposure. After 72 hours of exposure to paracetamol the relative gene expression was still significantly higher in treated plants. No induction of gene expression was observed one week after treatment (Fig. 3.25 b).

Up-regulation of GST-U5 in roots was also observed; it showed a maximum increase of 400 % of control after 168 hours of exposure (Fig. 3.25 a), whereas in leaves no significant changes in gene expression was observed (Fig. 3.25 b).

Expression levels for GST-U19 and GST-U24 were not affected by paracetamol treatment in root, while in leaf a strong down-regulation could be observed.

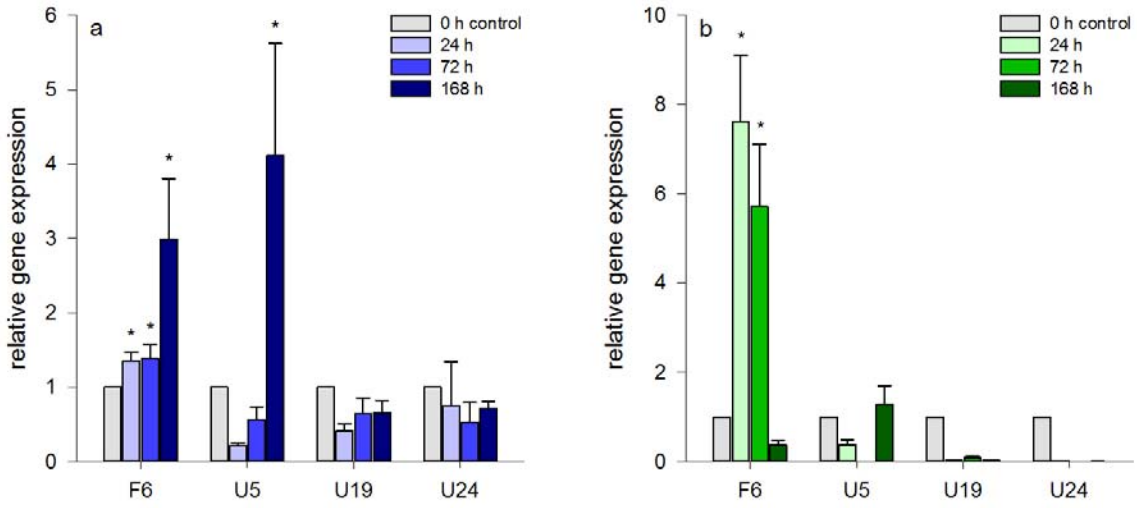


Figure 3.25 Relative gene expression changes of selected *Brassica* GST genes under 1 mg/L paracetamol exposure in roots (a) and leaves (b). GST expression levels were normalized to Actin and GAPDH housekeeping genes. Error bars represent ± S.D. of triplicate determinations.

3.2.6 Oxidative stress and enzymatic defense responses in *Brassica juncea*

1 mM paracetamol exposure

The plants exposed to 1 mM pharmaceutical showed visual stress symptoms; increasing amounts of bleaching and dot-like lesions on the adaxial side of the leaf were observed 72 hours after treatment; necrosis occurred after 7 days. Between 72 and 168 hours of exposure the roots began to develop a dark brownish color and no new lateral roots developed. All visual symptoms recorded pointed to strong oxidative stress reactions in the treated plants.

Correspondingly, ascorbate peroxidase showed a continuous decrease of activity in root tissues up to 50% of control after 168 hours exposure (Fig. 3.26 a), while in leaf samples increasing activity was observed, however without significance (Fig. 3.26 b).

Catalase activity in roots as well in leaf tissues strongly decreased to levels of less than 25% of control after 168 hours treatment (Fig. 3.26 c-d).

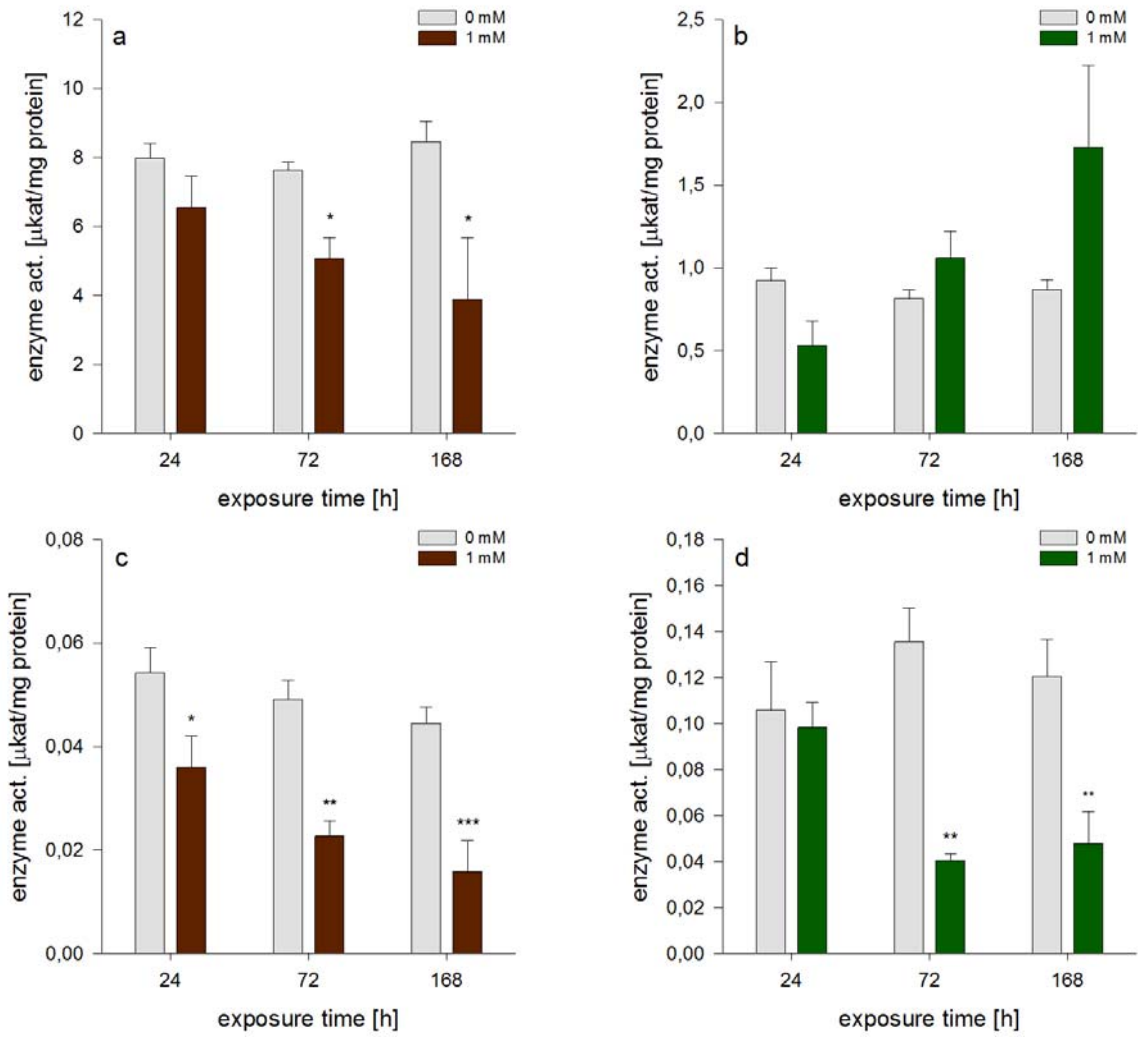


Figure 3.26 Specific enzyme activities in *Brassica* under 1 mM paracetamol exposure: APOX in roots and leaves (a-b), CAT in roots and leaves (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

Glutathione reductase showed significantly decreased enzyme activity similar to APOX and CAT in root (Fig. 3.27 a). In leaf a significant decrease in the enzyme activity (50% to control) was determined only after 24 hours exposure time (Fig. 3.27 b).

The POX activity showed slight increase in root after 24 hours, but after 3 days of exposure the enzyme activity decreased too (Fig. 3.27 c). Only in leaf tissues was a significantly and strongly increase in peroxidase activity determined over the time; 260% of control after 24 hours, 340% of control after 72 hours and 420% of control after 168 hours of exposure time (Fig. 3.27 d) were reached.

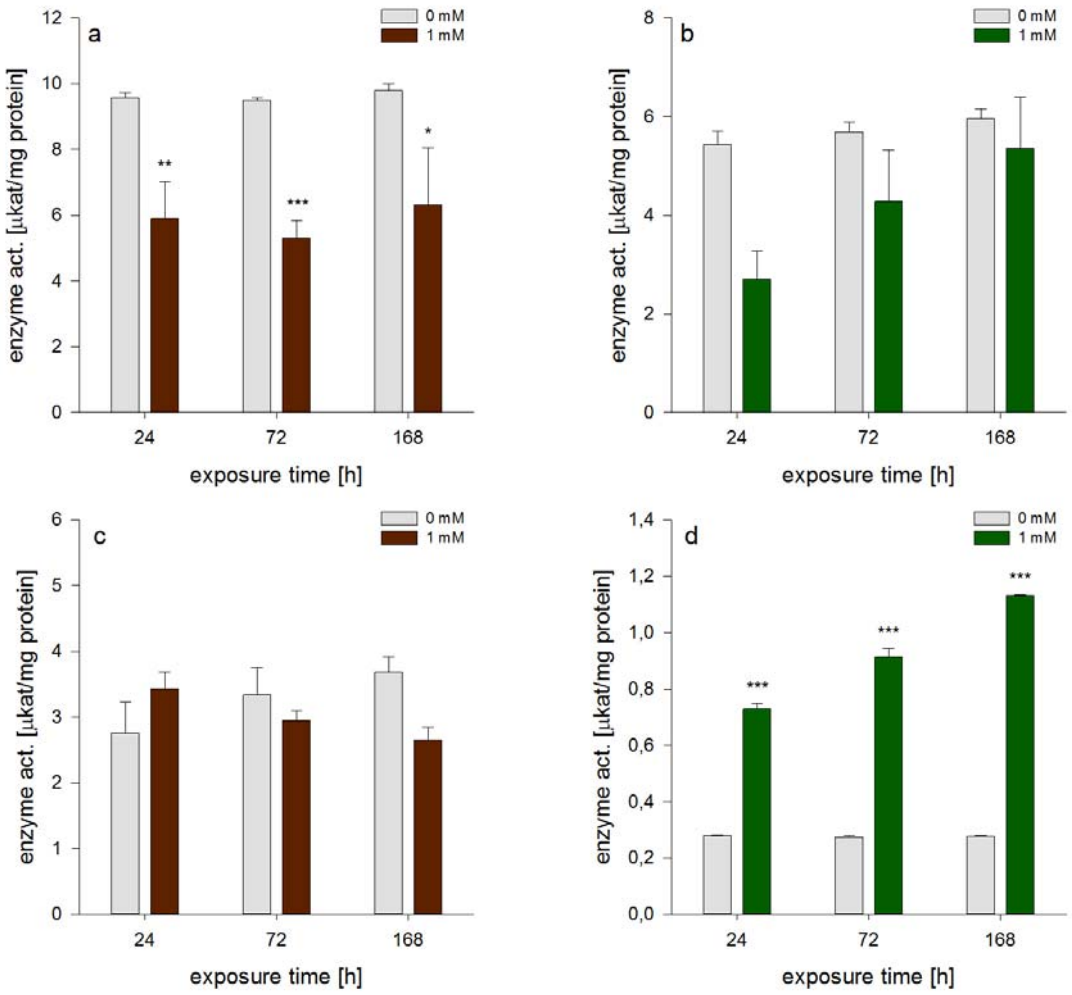


Figure 3.27 Specific enzyme activities in *Brassica* under 1 mM paracetamol exposure: GR in roots and leaves (a-b), POX in roots and leaves (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

1 mg/L paracetamol exposure

Brassica exposed to 1 mg/L paracetamol showed no visual symptoms during the experiments; no marked difference could be seen between treated and control plants. Similar to *Typha* plants, no remarkable CAT activity was detected.

APOX activity was decreased both in root and leaf tissues (Fig. 3.28 a-b); only 30% of control activity in leaf was detected after 24 and 72 hours of exposure. However after 168 hours the enzyme activity was 65% of control (Fig. 3.28 b).

Comparably, GR activity changed in a very similar way; decreased enzyme activity was observed in both tissues (Fig. 3.28 c-d). Strong loss of enzyme activity was measured in leaves 24 and 72 hours after treatment, i.e. only 27-28% of the control activity (Fig. 3.28 d) was left.

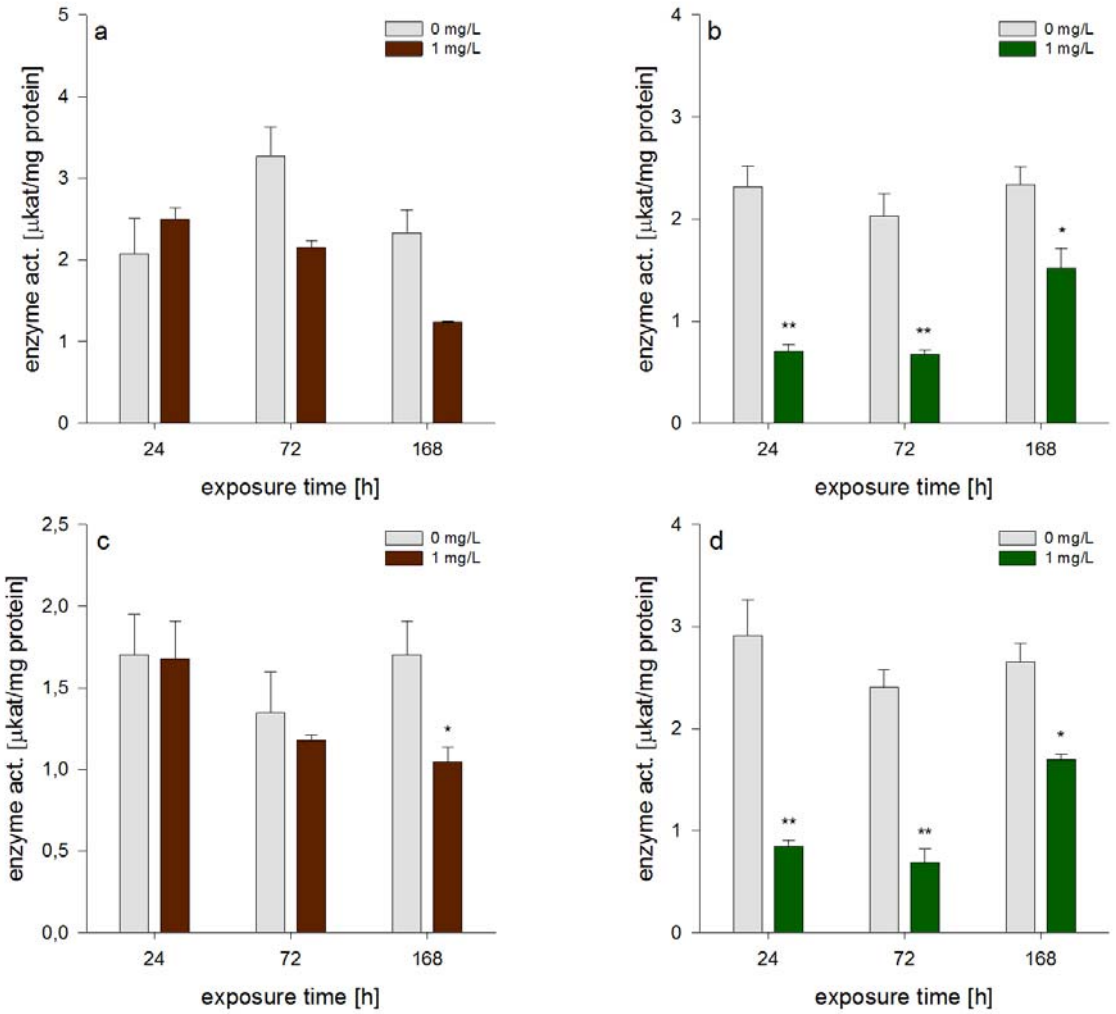


Figure 3.28 Specific enzyme activities in *Brassica* under 1 mg/L paracetamol exposure: APOX in roots and leaves (a-b), GR in roots and leaves (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01 vs. control.

Cytosolic POX activity

POX enzyme activity was determined in cytosolic and microsomal extracts of *Brassica* plants. In the cytosolic enzyme fraction the POX activity decreased strongly in roots as well in leaves. The strongest loss of enzyme activity (20% of control) in roots occurred after 7 days of exposure (Fig. 3.29 a), whereas in leaves 3 days after exposure only 15% of control activity was measured (Fig. 3.29 b).

Microsomal POX activity

The 1mg/L paracetamol treatment caused opposite effects on the microsomal enzyme fraction; increasing enzyme activity was observed over the experimental time in roots and reached its maximum after 168 hours at 190% of control activity (Fig. 3.29 c). In leaves a 7-fold activity increase was observed already 24 hours after treatment. At the end of the experiment the enzyme activity was still 480% of control (Fig. 3.29 d).

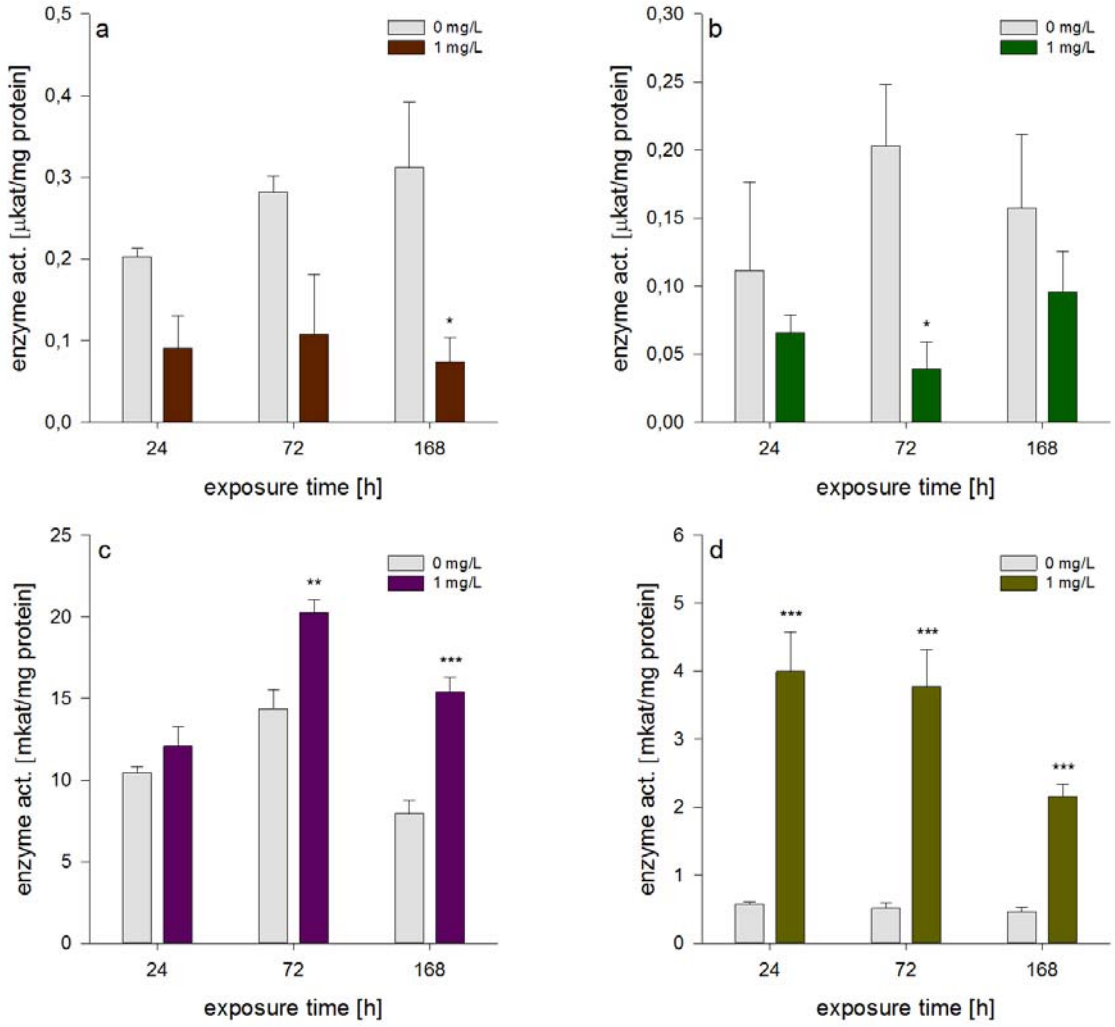


Figure 3.29 Specific POX enzyme activities in *Brassica* under 1 mg/L paracetamol exposure: cytosolic enzyme activity in roots and leaves (a-b), and microsomal enzyme activity in roots and leaves (c-d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

3.3 **Diclofenac uptake and detoxification in *Typha latifolia***

3.3.1 Uptake and translocation of diclofenac

To evaluate diclofenac detection in the plant and medium samples diclofenac standards were prepared in concentrations ranging from 1 – 0.01 mg/L in 3% acetonitrile with 0.1% formic acid and analyzed using tandem MS (MS/MS) in positive ionization mode of the LC-MS. The mass transition of 297 → 278 *m/z* was detected after 10.2 minutes retention time for diclofenac quantification (Fig. 3.30).

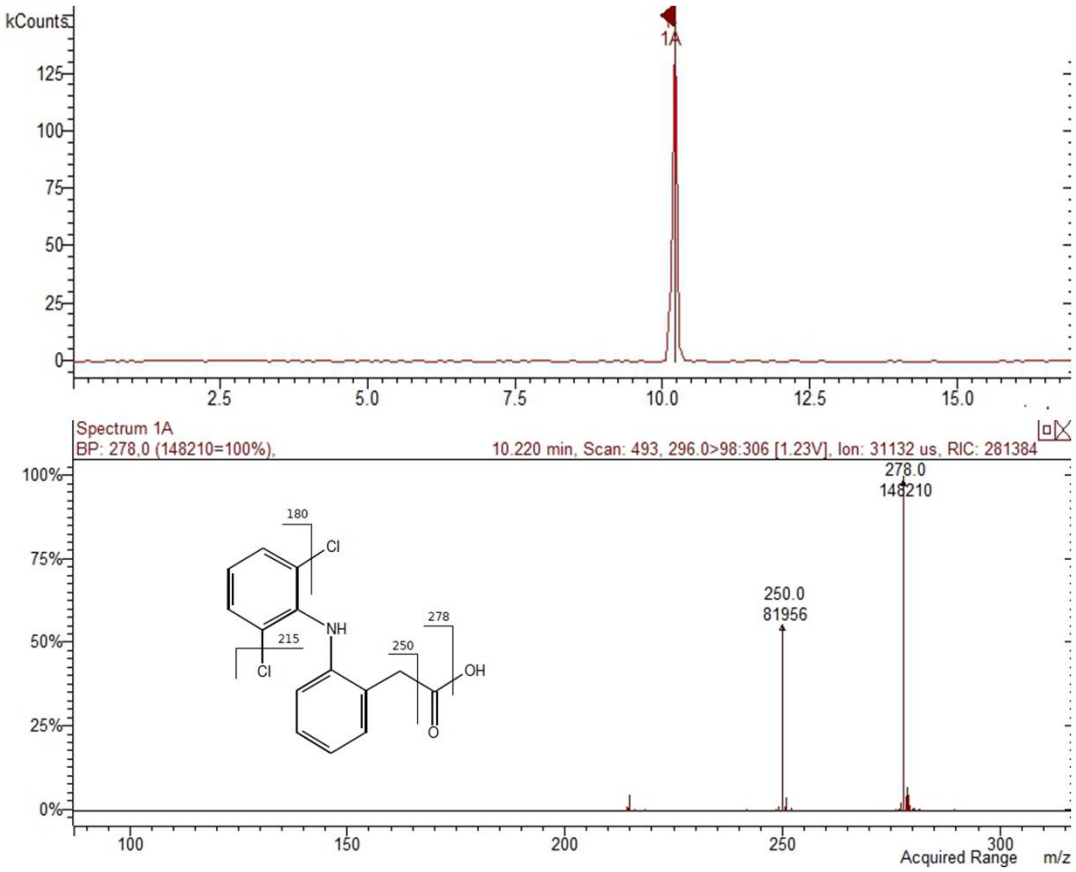


Figure 3.30 LC-MS/MS chromatogram and spectra of a diclofenac standard $(M+H)^+$ *m/z* 296 using the positive ionization mode.

For the uptake experiment *Typha* plants were exposed to 1 mg/L diclofenac for 30 days; root and shoot samples were collected after 1, 3, 7 and 30 days to analyze the pharmaceutical uptake. After 1 day of exposure diclofenac was already detected in both tissues; 0.2 µg/g FW in roots and 0.013 µg/g FW in shoots (Fig. 3.31). After 3 days a lower diclofenac concentration was detected in the root (0.12 µg/g FW), which stayed at this level until the end of the experiment.

The concentration of diclofenac in shoots remained at the same level during 30 days of exposure and showed its maximum of 0.02 µg/g FW 7 days after treatment. The ten-fold lower and constant drug concentration in shoots points to moderate transport to the upper part of the plants and indicates low metabolic activity.

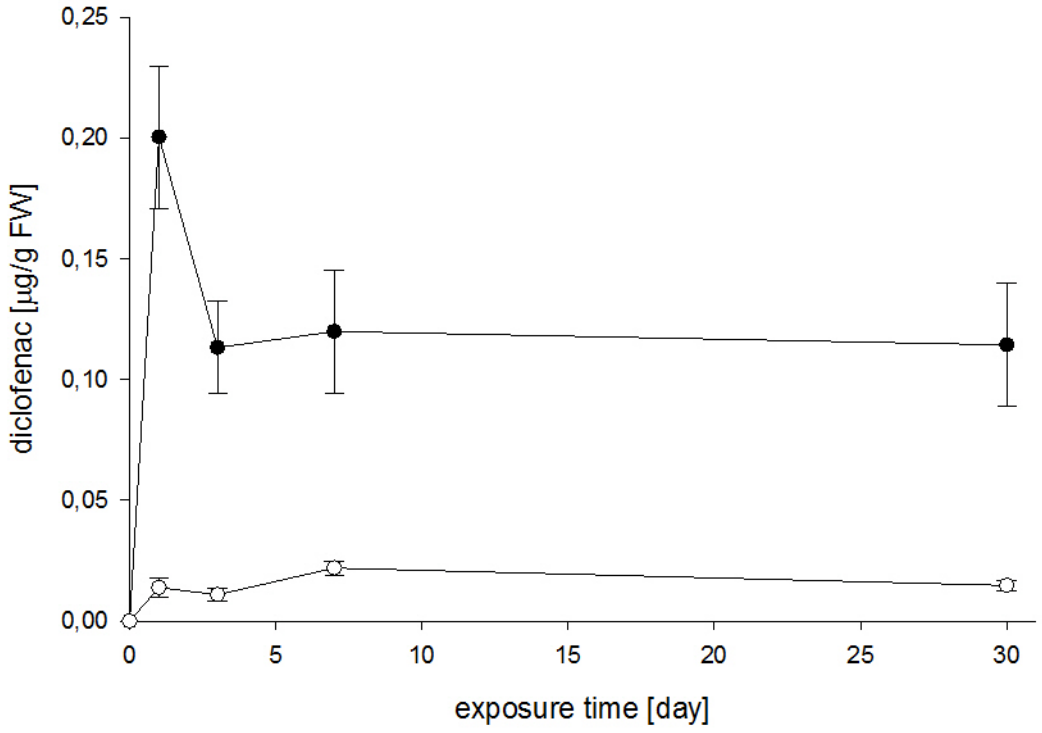


Figure 3.31 Diclofenac uptake and transport in *Typha* root (●) and leaf (○) tissues after 1 mg/L pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

3.3.2 Short term uptake of diclofenac from culture medium

Similar to the paracetamol experiment and to determine possible abiotic degradation and surface adsorption effects, aliquots of the culture medium were collected. The experiment was carried out using 1 mg/L and 10 mg/L diclofenac concentrations.

Diclofenac was detected in the incubation medium in the presence of *Typha latifolia* roots and in a control medium without plants as well as in the plant roots. The control medium containing 1 mg/L diclofenac showed a slightly decreased drug concentration of 0.8 mg/L 24 hours after incubation, which points to a probable concentration loss due to adsorption effects in the range of 0.1-0.2 mg/L (Fig. 3.32 a, solid line). In case of the 10 mg/L treatment this effect was not observed (Fig. 3.32 b, solid line).

The medium samples of incubations in the presence of root segments had strongly decreased diclofenac content; during the first four hours of incubation the drug concentration dropped by 52% in the 1 mg/L treatment (Fig. 3.32 a, dotted line) and by 73% in the 10 mg/L treatment (Fig. 3.32 b, dotted line). In parallel, 0.16 mg/L diclofenac uptake from 1 mg/L treatment (Fig. 3.32 a, dashed line) and 1.1 mg/L diclofenac uptake from 10 mg/L treatment (Fig. 3.32 b, dashed line) was detected in the exposed roots.

Table 3.4 represents the calculated surface adsorption and the uptake rates in the root segments. The uptake in the roots accounts in both cases for about 10% of the concentration decrease in the medium. It is suggests that adsorption onto the root surface is the main process leading to diclofenac concentration decrease; the calculated surface adsorption on the root is 50% in case of 1 mg/L exposure and 25% in case of 10 mg/L exposure 24 hours after incubation (Table 3.4).

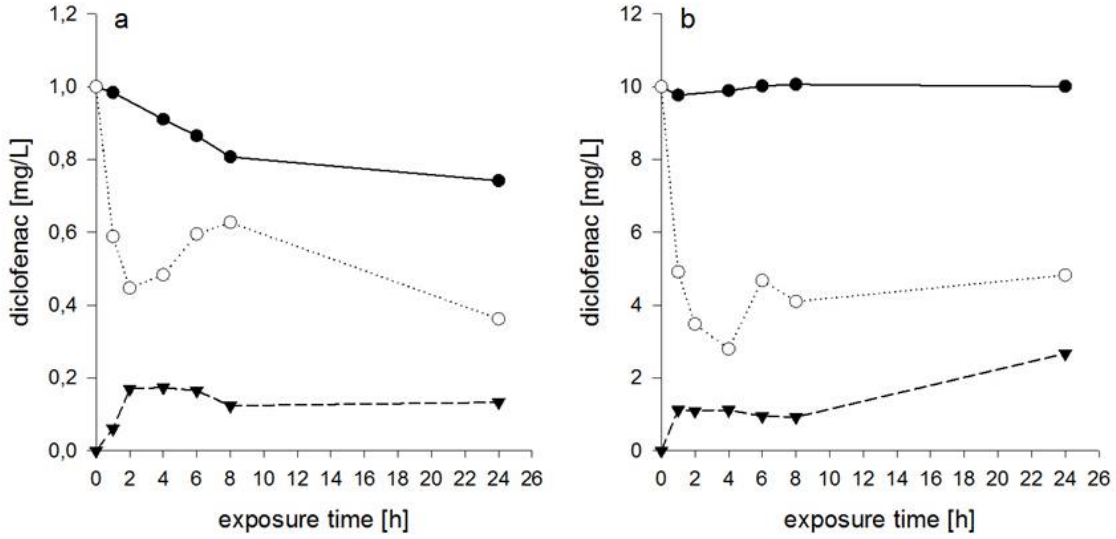


Figure 3.32 Diclofenac concentration changes in medium containing 1 mg/L diclofenac (a) and 10 mg/L diclofenac (b) in the absence of root material (solid line ●), in the presence of root material (dotted line ○) and in the root tissues (dashed line ▼).

Table 3.4 Fate of diclofenac from the culture medium due to surface adsorption and uptake by *Typha* roots during 24 hours. N.B.: The adsorption could not be calculated on a surface basis because roots did not represent ideal cylindrical form.

time	1 mg/L			10 mg/L		
	surface adsorption (mg)	uptake (mg/g FW)	uptake rate (mg/min/g FW)	surface adsorption (mg)	uptake (mg/g FW)	uptake rate (mg/min/g FW)
1 h	0.35	0.06	0.001	3.96	1.12	0.02
4 h	0.34	0.173	0.0007	6.08	1.116	0.005
6 h	0.34	0.064	0.0002	4.84	0.485	0.001
8 h	0.29	0.123	0.0002	4.97	0.925	0.002
24 h	0.50	0.133	0.0000	2.51	2.667	0.002

3.3.3 Identification of diclofenac metabolites

The major diclofenac metabolites in humans are hydroxylated diclofenac products, mainly 4'-OH diclofenac and acyl glucuronide of diclofenac. Glutathione conjugated metabolites are also described in mammals (Tang et al., 1998). In plants, diclofenac metabolism was first described by Huber (2011): 4'-OH diclofenac and diclofenac-glucopyranoside conjugate were identified in diclofenac treated tissue culture of horseradish and in barley plants.

4'-OH diclofenac was identified after MS/MS detection of the fragmentation of $(M+H)^+$ *m/z* 312 using positive ionization mode. The main fragments are *m/z* 294 and *m/z* 266 (Fig. 3.33 a). MS/MS spectra comparison of analytical standard and plant samples confirmed the formation of 4'-OH diclofenac in plants.

Fragmentation of the pseudo molecular ion *m/z* 474 → 312 was detected to determine the diclofenac-glucopyranoside conjugate. The MS/MS spectra of the conjugate shows the characteristic fragments *m/z* 312, *m/z* 294 and *m/z* 266 (Fig. 3.33 b). The determination of the conjugate has been confirmed by incubation with *β*-glucosidase, which leads to the cleavage of the conjugate.

In the full scan analysis of root samples the signal of *m/z* 617 was found, which corresponds to the glutathione conjugated diclofenac metabolite (Tang et al. 1998). The comparison of the MS/MS spectra of the plant samples with the spectra published in the literature showed the same fragmentation pattern with the characteristic product ions *m/z* 342, *m/z* 324, *m/z* 488 and *m/z* 542. The MS/MS spectra of the plant samples showed the same fragmentation pattern (Fig. 3.33 c), therewith it is the first evidence of the existence of the glutathione dependent metabolism of diclofenac in plants.

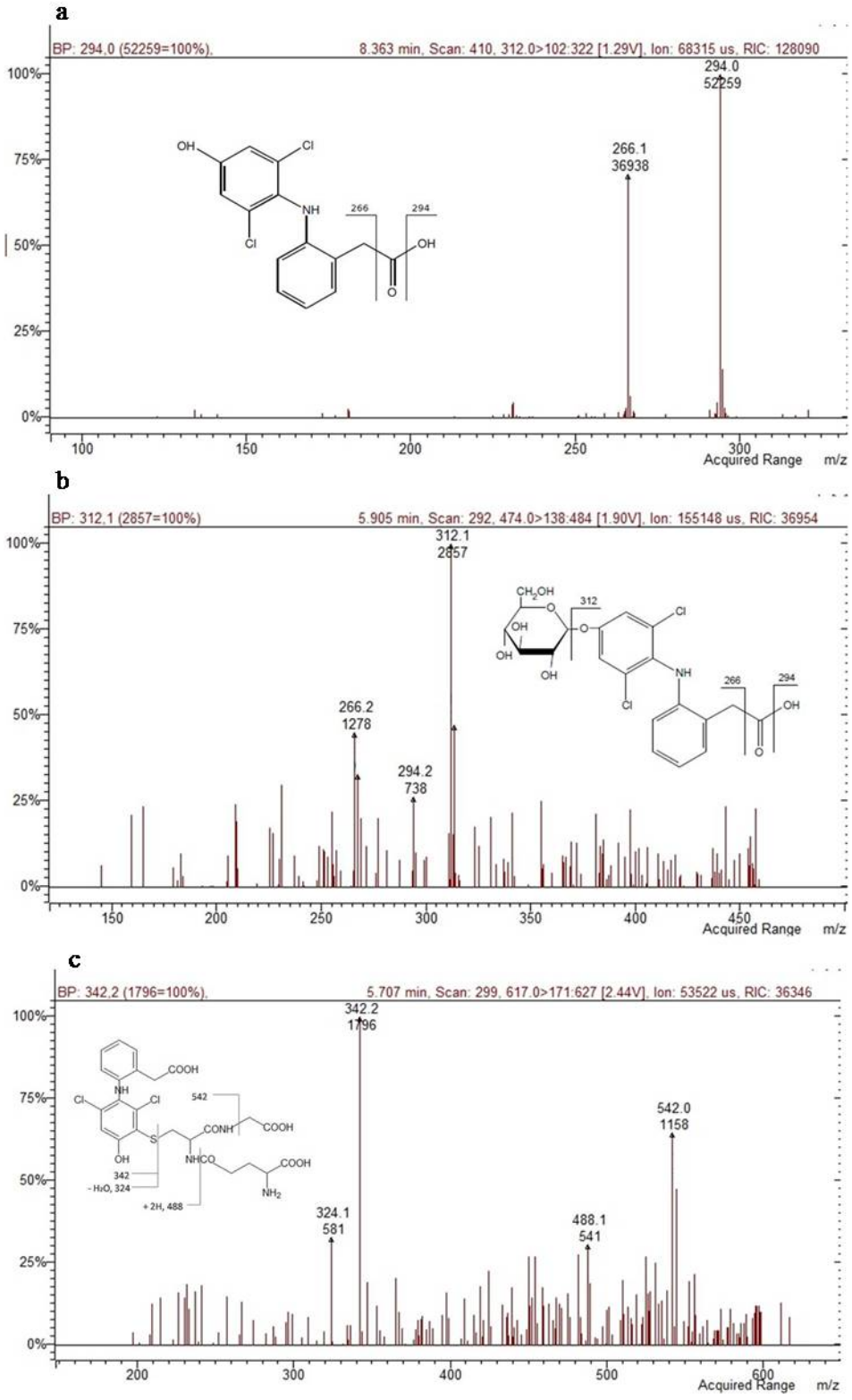


Figure 3.33 MS/MS spectra of the 4'-OH diclofenac $(M+H)^+$ *m/z* 312 (a), diclofenac-glucopyranoside $(M+H)^+$ *m/z* 474 (b) and OH-diclofenac-GS conjugates $(M+H)^+$ *m/z* 617 (c) in *Typha latifolia* root tissues using positive ionization mode.

Rapid uptake and metabolism of diclofenac was observed in *Typha* roots after exposure to 1 mg/L of the drug; 4'-OH diclofenac, the phase I detoxification product of the drug, was detected already 24 hours after treatment in higher amount than diclofenac itself. The phase II detoxification product diclofenac-glucopyranoside was also detected in smaller amount; 80% of the metabolized diclofenac was present in the form of OH-diclofenac and 20% in the form of a sugar conjugate (Fig. 3.34 a).

In all experiments, the relative amount of OH-diclofenac was decreased after three and seven days of exposure and stayed at this level until the end of the experiment (about 36.2% of the metabolized diclofenac). In contrary, the relative amount of glucopyranoside conjugate increased continuously; 30 days after treatment 67.3% of the metabolized diclofenac was present in this form in the plant roots (Fig. 3.34 a).

The diclofenac-glutathione conjugate was first detected after 3 days of exposure in very small amounts and showed not further increase; at the end of the experiment 2% of the metabolized drug was detected in this form (Fig. 3.34 a).

Although a small amount of diclofenac was detected in shoots (4% of the root amount after one day, 22% of the root amount seven days after treatment), only a small amount was metabolized to 4'-OH-diclofenac, due to phase I process hydroxylation. Although diclofenac was decreased in leaves after seven days till the end of the experiment, and the OH-diclofenac had also disappeared, no other metabolic product was detected in the upper part of the plants (Fig. 3.34 b). This effect could be explained by the biomass gain during the one month treatment time, which leads to a decrease of the relative amount of diclofenac, calculated on a fresh weight basis.

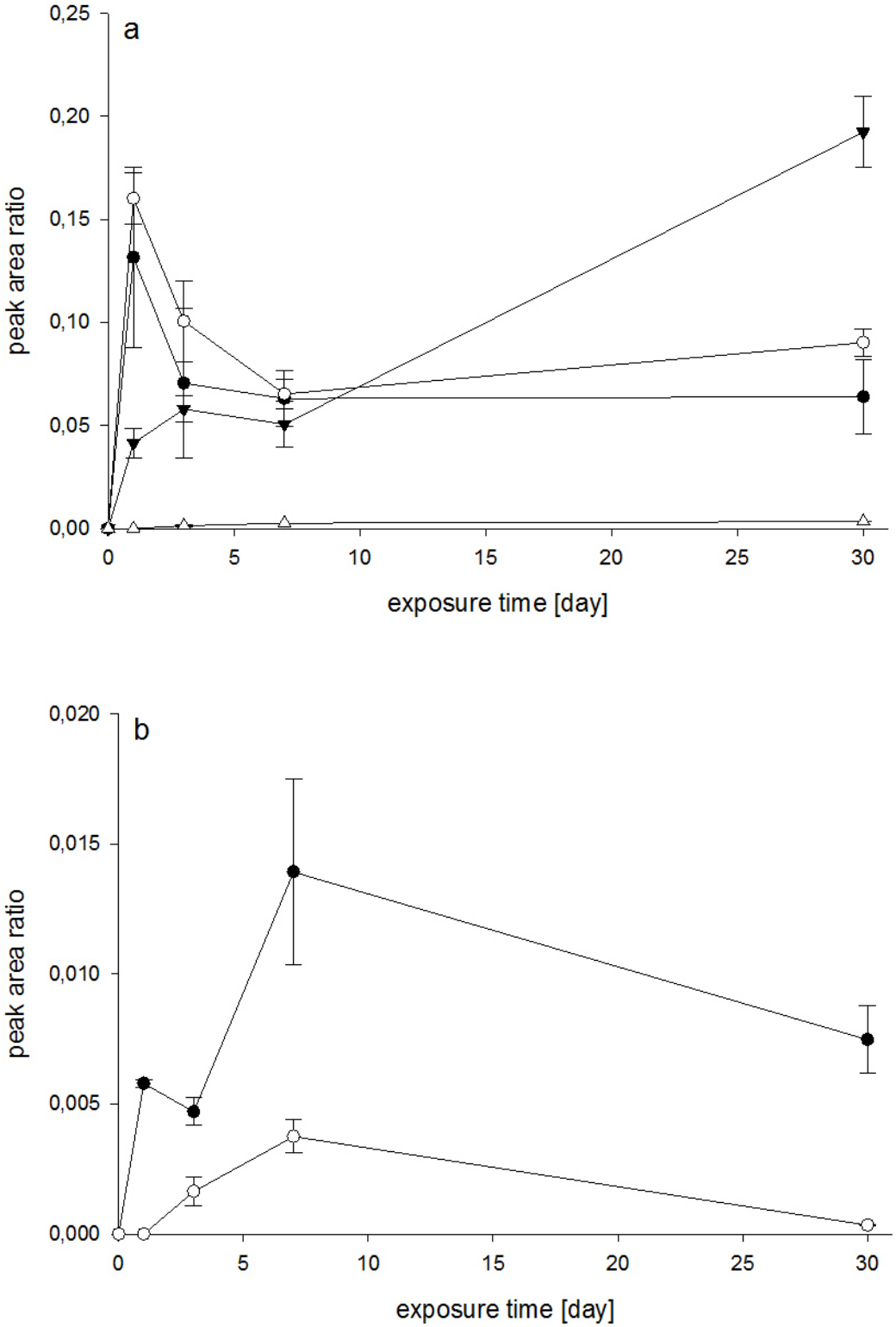


Figure 3.34 Relative amounts of diclofenac (●), 4'-OH diclofenac (○), diclofenac-glucopyranoside (▼) and OH-diclofenac glutathione (∆) conjugates in *Typha* root (a) and shoot (b) tissues after 1 mg/L pharmaceutical exposure.

3.3.4 Effect of diclofenac on the Glycosyltransferase activity in *Typha latifolia*

Glycosyltransferase activity was determined using the same substrates as described in chapter 3.1 and 3.2 during the paracetamol experiments: the xenobiotic compound TCP, and two flavonoids, quercetin and kaempferol, were used in the assay. Table 3.5 represents the activities of GT in *Typha* root and shoot tissues for these metabolites.

In roots no enzyme activities were detected with the substrate TCP, while in leaves increased GT activity was detected after exposure to diclofenac. The activity change reached its maximum already one day after pharmaceutical exposure (190% above control) followed by decreasing enzyme activity. After one month, the activity had decreased to 41% of control.

Using quercetin and kaempferol as substrates, strongly increased GT activity was observed in plant roots. Elevated GT activity was also detected in plant shoots, but not as high as in the root samples. GT-quercetin activity was increased to 275% of control one day after diclofenac treatment, showed increasing activity during the first week and reached its activity maximum after seven days of exposure at 647% of control level. However the enzyme activity decreased after 30 days of exposure, but remained still 2.5-fold higher than in control plants. The same enzyme activity changes were detected when kaempferol was used as substrate; GT activity was increased during the first seven days in the treated plant roots and reached its maximum with a more than six-fold activity increase at the end of the first week after treatment. After 30 days of exposure GT activity was still higher than control, but only at 253%.

In the plant leaves, similar to GT-TCP activity, elevated GT-quercetin and GT-kaempferol activities were detected after diclofenac exposure. Nevertheless, the changes were not as strong as in the roots; 1.6-fold increase after three days of exposure with quercetin and 1.7-fold increase after one day treatment with kaempferol was the highest enzyme activity change in the treated leaves.

The effect of the diclofenac treatment was an activation of the GT enzymes, both in roots and shoots; however the conjugation product was only detected in roots. The increased activity in the shoot tissues refers to detoxification via glucose conjugation, but on the score of the LC-MS measurements this process seems to be running only in the plant roots.

Table 3.5 Glycosyltransferase activity in *Typha* control and 1 mg/L diclofenac treated plants. Changes in enzyme activity are also represented as % of control.

GT activity [pkat/mg protein]						
	Roots			Shoots		
Time [day]	TCP			TCP		
	control	diclofenac	control%	control	diclofenac	control%
1	n.d.	n.d.	n.d.	0.8±0.03	1.69±0.14	190%
3	n.d.	n.d.	n.d.	1.01±0.02	1.38±0.7	141%
7	n.d.	n.d.	n.d.	0.87±0.03	1.27±0.14	145%
30	n.d.	n.d.	n.d.	1.2±0.02	0.52±0.08	41%

	quercetin			quercetin		
	control	diclofenac	control%	control	diclofenac	control%
1	1.92±2E-04	4.33±1E-03	275%	1.57±3E-03	1.6±4E-04	83%
3	1.4±4E-04	3.68±1E-03	509%	1.53±5E-03	2.37±2E-04	168%
7	0.97±6E-05	8.83±6E-03	647%	2.2±1E-03	1.28±5E-04	132%
30	1.33±4E-04	7.72±1E-03	256%	2.01±1E-03	1.36±6E-05	102%

	kaempferol			kaempferol		
	control	diclofenac	control%	control	diclofenac	control%
1	0.85±6E-04	4.02±5E-04	369%	1.1±3E-03	1.49±1E-04	173%
3	1.52±1E-04	3.11±8E-04	403%	1.65±4E-04	1.99±1E-04	131%
7	1.46±2E-05	7.04±4E-03	678%	1.6±1E-03	0.87±6E-04	59%
30	1.33±3E-04	6.00±1E-03	253%	1.6±8E-04	1.34±3E-03	100%

3.3.5 Effect of diclofenac on the Glutathione S-transferase activity in *Typha latifolia*

Glutathione S-transferase activity was determined in cytosolic and microsomal extracts of *Typha* plants after 1 mg/L diclofenac exposure, using different substrates (CDNB, Fluorodifen and pNpa) to characterize the role of the different GST isoforms in the detoxification processes and stress reactions.

Cytosolic GST activity

In root tissues the cytosolic GST-CDNB activity was significantly increased; 215% of control after 3 days, 154% of control after 7 days and 282% of control after 30 days diclofenac exposure was detected (Fig. 3.35 a). Similar to roots, increased GST-CDNB activity was detected in the shoot tissues; it reached its maximum after seven days of exposure at 210% of control, but 30 days after treatment the activity had decreased to the control level (Fig. 3.35 b).

GST activity in roots showed already one day after treatment slightly increased activity with the other two substrates fluorodifen and pNpa. GST-fluorodifen activity increased to 134% of control after one day of exposure and showed elevated activity during the first week. It's the maximum level was reached after 7 days (180% of control) of exposure. After 30 days the activity increase returned to the control level (Fig. 3.36 a). In shoots GST-fluorodifen activity was inhibited, however the results of three biological replications were not sufficient to reach significant changes (Fig. 3.36 b).

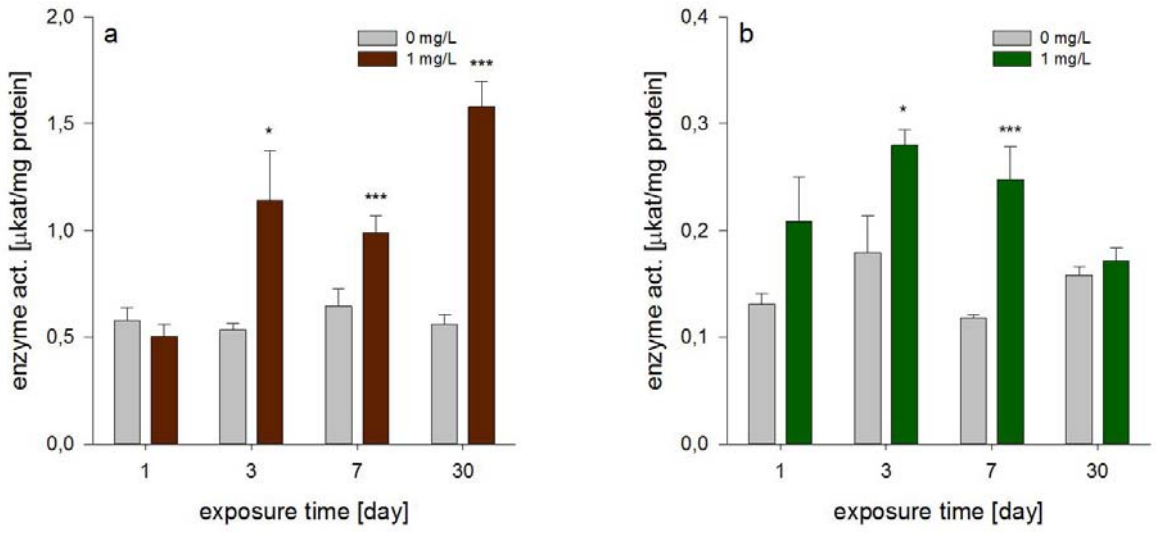


Figure 3.35 GST-CDNB activity in *Typha* roots (a) and shoots (b) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, ***p<0.001 vs. control.

When pNpa was used as a substrate, GST activity showed a slight increase (117% of control) in roots after one day of exposure, but after that no significant enzyme activation was determined during the first week. Only 30 days after treatment a slight increase (140% of control) was observed (Fig. 3.36 c). In leaves a two-fold enzyme activity increase was observed after one day of exposure, but after that, similar to GST-fluorodifen activity, no significant changes were observed (Fig. 3.36 d).

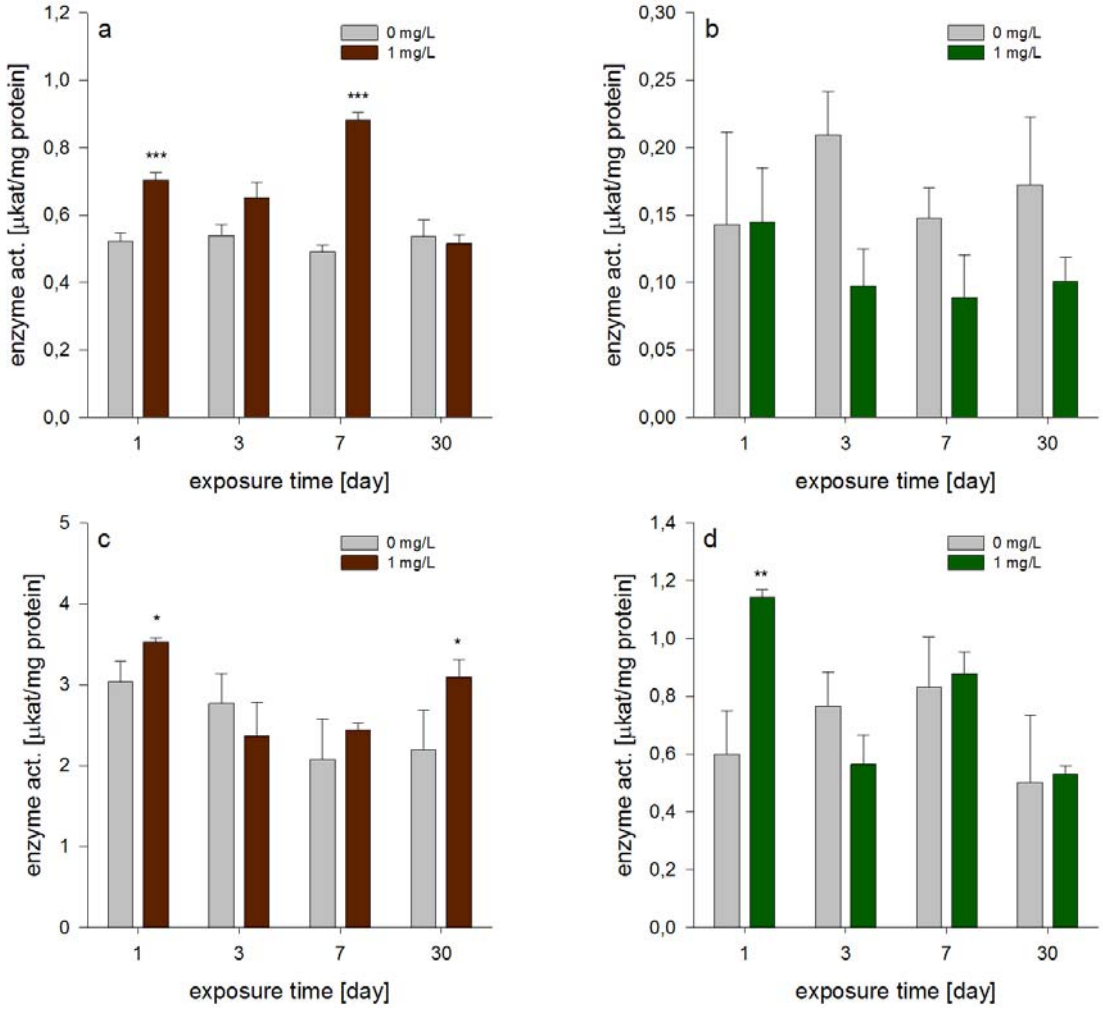


Figure 3.36 GST-fluorodifen activity in *Typha* roots and shoots (a-b) and GST-pNpa activity in *Typha* roots and shoots (c-d) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

Microsomal GST activity

GST activity was determined in microsomal extracts of *Typha* plants using the same substrates as in case of the cytosolic extracts. In roots GST showed increased activity after diclofenac exposure using CDNB as substrate; compared to the cytosolic GST-CDNB activity in the microsomal fraction the activity increased to 190% of control already one day after treatment. The largest activity increases were detected 7 days (325% of control) and 30 days (200% of control) after treatment (Fig. 3.37 a). Activity increase was detected in shoots too; 210% of control level already one day after diclofenac exposure. After that enzyme activity decreased to the control level 3 and 7 days after treatment and decreased below the control activity level at the end of the experiment (Fig. 3.37 b).

Increased enzyme activity was observed in roots using fluorodifen as substrate; in this case the highest activity changes were observed after one day (134% of control) and three days (210% of control) of exposure. Decreased, but still elevated, activity (140% and 150%) compared to control was observed 7 and 30 days after exposure (Fig. 3.37 c). In leaves the same enzyme activity changes were observed as in case of CDNB substrate; after a short time induction (180% of control after one day of exposure) the GST activity was back to the control level till the end of the experiment (Fig. 3.37 d).

Using pNpa as substrate, microsomal GST showed increased activity both in roots and shoots over the experiment. The enzyme activity reached its maximum in roots (Fig. 3.37 e) as well as in leaves (Fig. 3.37 f) after three and seven days of exposure with roughly the same activity increase (about 50%) compared to control.

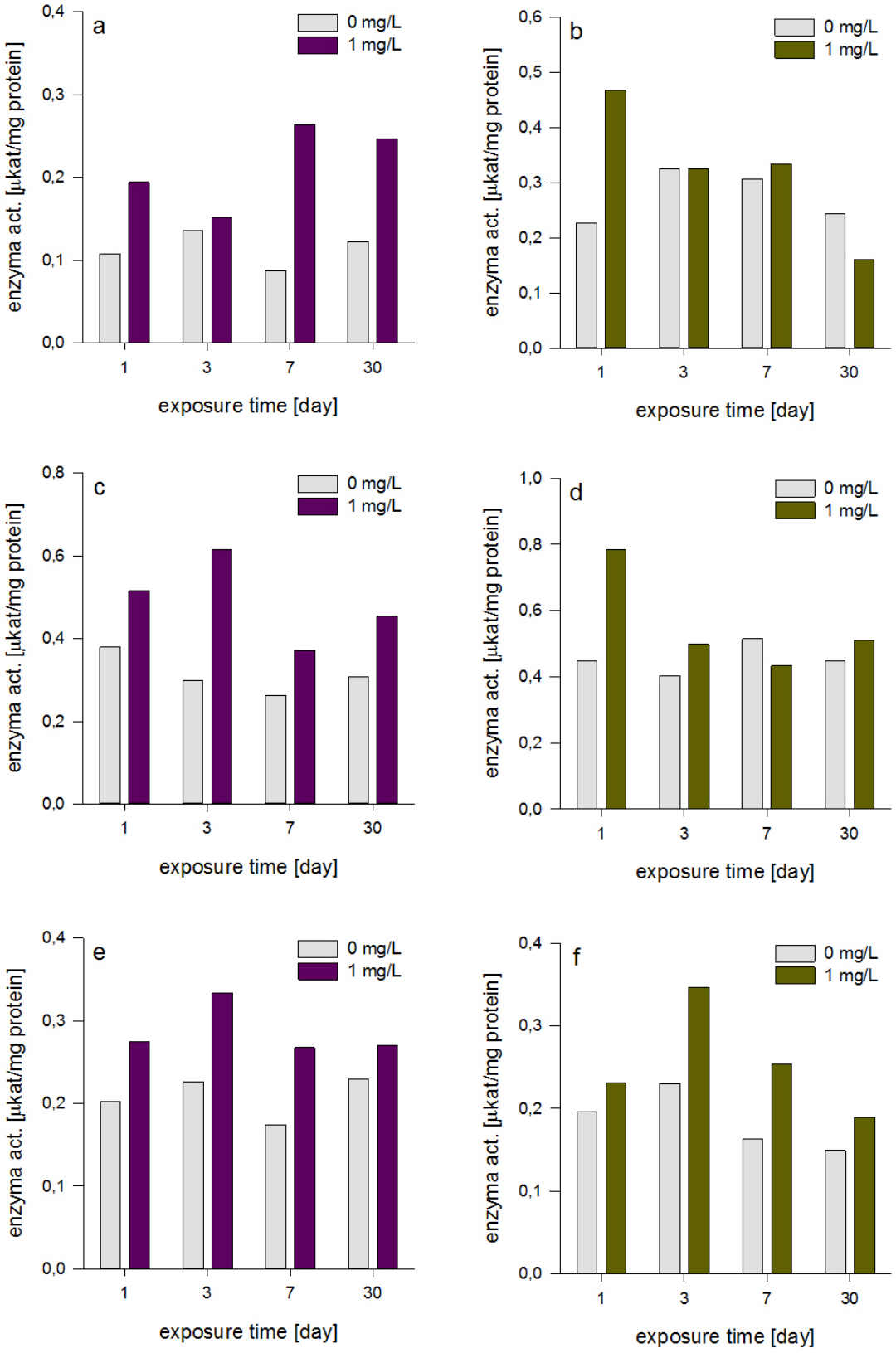


Figure 3.37 Microsomal GST activity in *Typha* plants under 1 mg/L diclofenac exposure using different substrates to detect the enzyme activity. GST-CDNB activity in roots (a) and shoots (b), GST-fluorodifen activity in roots (c) and shoots (d) and GST-pNpa activity in roots and shoots (d). Fresh biomass of three biological replicates was pooled during the extraction to reach the required fresh weight.

3.3.6 Oxidative stress and enzymatic defense response in *Typha latifolia*

The effect of diclofenac exposure on the antioxidative defense enzymes in *Typha* plants was investigated by measuring the enzyme activity of APOX and GR, two key enzymes in the Halliwell-Asada pathway.

In roots, neither Ascorbate peroxidase, nor Glutathione reductase showed clear activation after 1 mg/L diclofenac treatment. Even significantly decreased APOX activity (38% of control) was observed at the end of the experiment, after 30 days of exposure (Fig. 3.38 a). Whilst changes in root enzymes were not significant, in shoots the APOX activity increased to 280% of control after one day and 170% of control after seven and thirty days of exposure (Fig. 3.38 b).

Glutathione reductase activity was only slightly increased in roots 30 days after treatment (Fig. 3.38 c), while in shoots the enzyme activity increased to 150% of control after the first day of exposure (Fig. 3.38 d). After that time no further significant activity increase was observed.

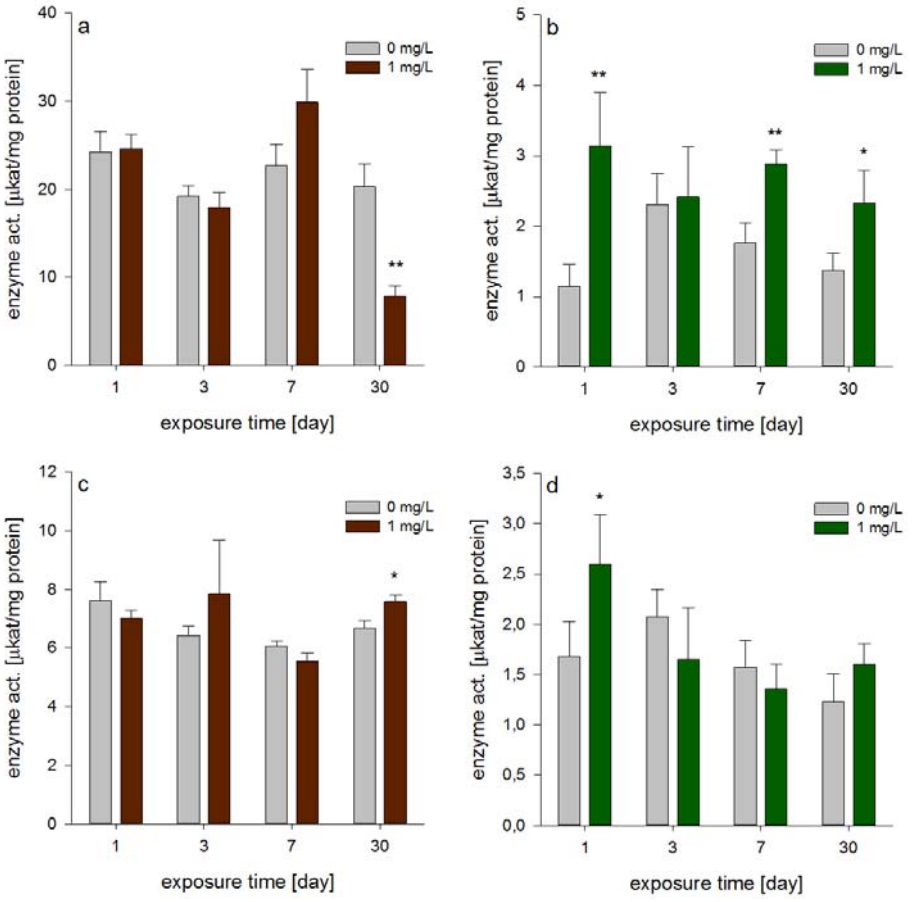


Figure 3.38 Specific enzyme activities in *Typha* under 1 mg/L diclofenac exposure: APOX in roots and shoots (a-b), GR in roots and shoots (c-d). Error bars represent ± S. D. of 3 biological replicates. *p<0.05, **p<0.01 vs. control.

Elevated POX activity has been frequently described under different biotic and abiotic stress conditions and commonly been explained as a general stress marker in plants due to its elimination role in H_2O_2.

Cytosolic POX activity

POX activity in the cytosolic fraction of *Typha* roots increased to 230% of control during the first three days of exposure and reached its maximum one week after treatment at 250% of control. At the end of the experiment the enzyme activity induction was lost and activities returned to the control level (Fig. 3.39 a). In plant leaves only a transient 2.3-fold enzyme activity increase was detected after the first day of diclofenac exposure. Subsequently POX activity even decreased compared to control plants (Fig. 3.39 b).

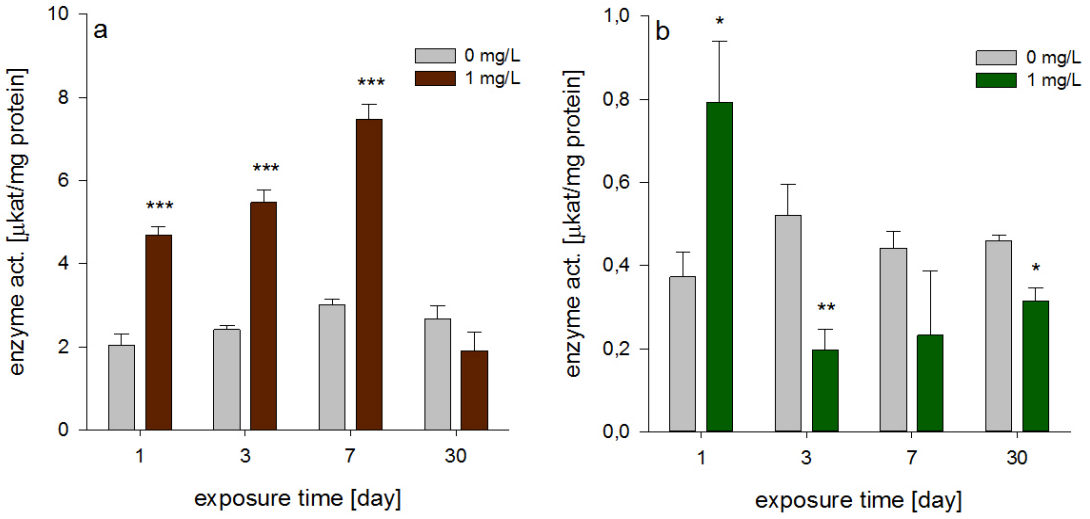


Figure 3.39 Cytosolic Peroxidase activity in *Typha* roots (a) and shoots (b) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, **p<0.01, ***p<0.001 vs. control.

Microsomal POX activity

Similar to cytosolic GST activity, POX activity in roots continuously increased to 230% of control level during the first week after treatment (Fig. 3.40 a). POX activity in plant leaves showed similar trends like the cytosolic enzymes; however in this case the loss of enzyme induction proceeded slower. From 230% of control activity after one day it decreased to 150% of control after 3 days of exposure and was followed by further decrease till the end of the experiment (Fig. 3.40 b).

In roots the same cytosolic and microsomal POX reaction was observed, but at day 3 microsomal POX remained induced, whereas cytosolic POX activity decreased already.

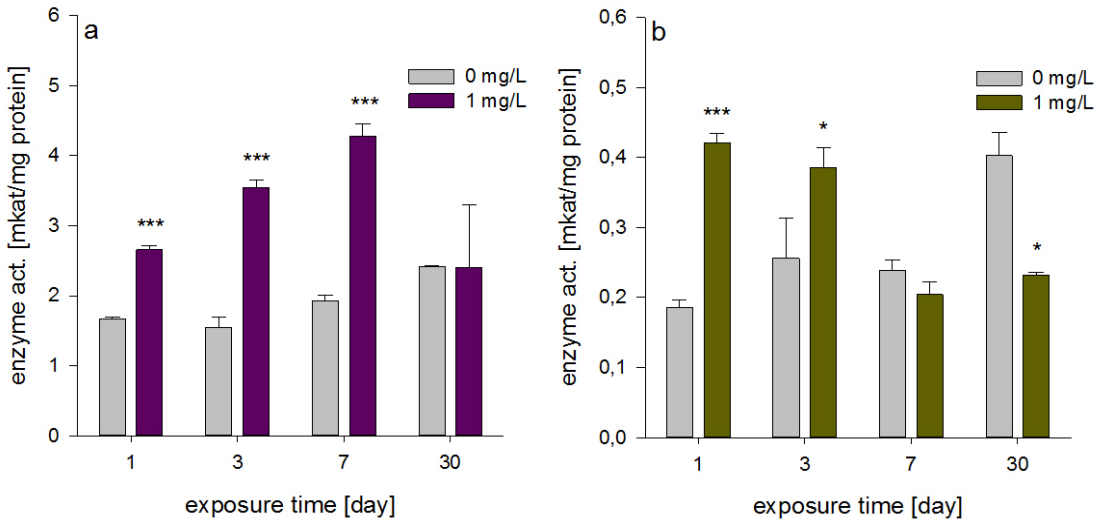


Figure 3.40 Microsomal Peroxidase activity in *Typha* roots (a) and shoots (b) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, ***p<0.001 vs. control.

3.4 **Diclofenac uptake and detoxification in *Brassica juncea***

3.4.1 Diclofenac uptake, translocation and metabolism in *Brassica juncea*

Diclofenac uptake and metabolism was investigated in Indian mustard plants exposed to 1 mg/L pharmaceuticals for one week. Already 24 hours after treatment 0.65 µg/g FW diclofenac was detected in roots and 0.7 µg/g FW after 3 days, which was the maximal uptake rate recorded during the experiment. After that time the detected diclofenac concentration dropped to 0.2 µg/g FW (Fig. 3.41 a). This strong decrease points to running metabolic processes in roots, since it cannot be covered by translocation to the upper parts of the plants.

Although diclofenac was detected in leaves after 24 hours of exposure, it was only 0.15% of the diclofenac detected altogether in both tissues. One week after treatment 0.2% of the determined diclofenac amount (1.6 ng/g FW) was transported to the leaves (Fig. 3.41 b).

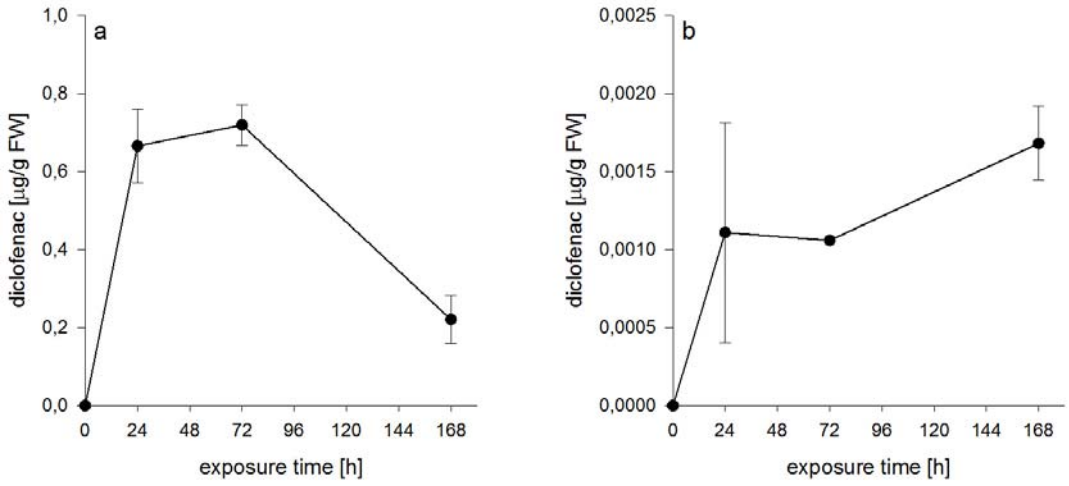


Figure 3.41 Diclofenac uptake in *Brassica* roots (a) and transport to leaves (b) after 1 mg/L pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

3.4.2 Diclofenac metabolites in Brassica

The same metabolic products were identified in *Brassica* and in *Typha* plants; the Phase I detoxification product 4'-OH diclofenac and Phase II metabolites diclofenac-glucopyranoside and OH-diclofenac glutathione conjugate were found.

All of the 3 metabolic products were detected after 24 hours of diclofenac treatment in the roots, however in very small amount compared to the amount of the parent compound itself; only 3% of the detected masses were metabolized diclofenac (Fig. 3.42). Over time the relative amount of metabolic products increased continuously and after one week of exposure 69% of the metabolized diclofenac was present in form of diclofenac-glucopyranoside (Fig. 3.42 ▼), 30% as 4'-OH diclofenac (Fig. 3.42 ○) and only 1% as OH-diclofenac glutathione conjugate (insert in Fig. 3.42). During the whole course of the experiment the relative amount of glucosyl-conjugate was twice as much as the glutathione conjugate and hence seems to be the main product in the detoxification pathway.

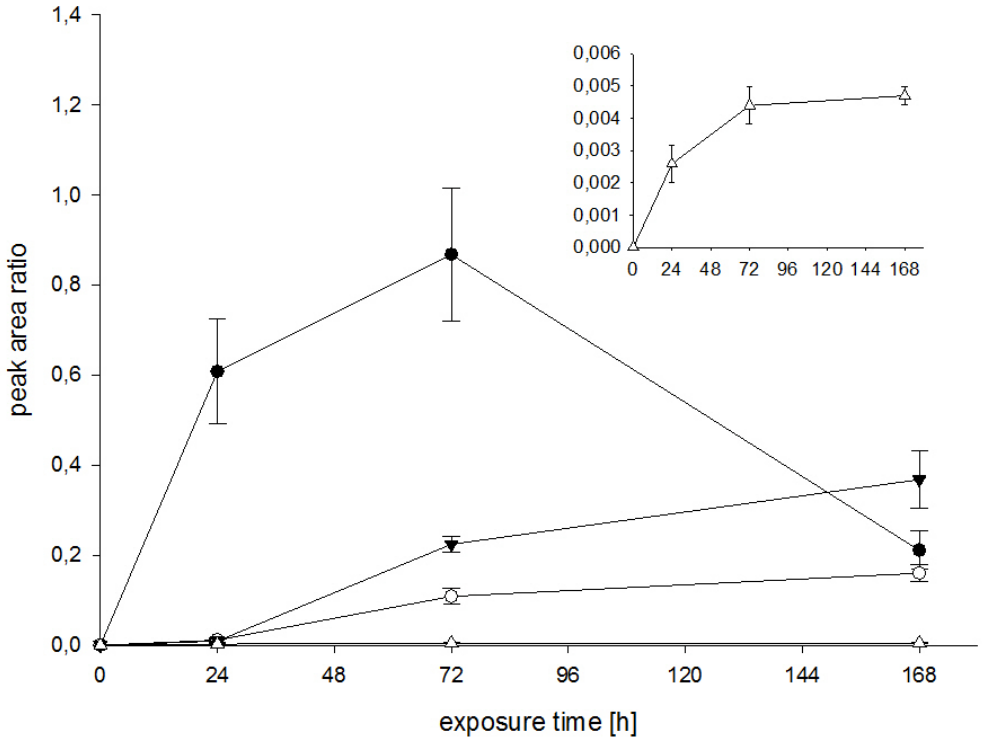


Figure 3.42 Relative amounts of diclofenac (●), 4'-OH diclofenac (○), diclofenac-glucopyranoside (▼) and OH-diclofenac glutathione (∆) conjugates in *Brassica* roots after 1 mg/L pharmaceutical exposure. The insert depicts the relative amount of OH-diclofenac glutathione. Error bars represent standard deviation of 3 biological replicates.

In leaves two of the metabolic products were identified; 4'-OH diclofenac and diclofenac-glucopyranoside, but not the glutathione conjugate. Only diclofenac-glucopyranoside was detected 24 hours after treatment, at concentrations close to the amount of diclofenac itself. Finally its amount was decreased to 50% at the end of the experiment, whilst the uptake of diclofenac was still increasing. After one week of exposure the glucose conjugate accounts for 13% of the metabolized drug (Fig. 3.43 ▼).

Although 4'-OH diclofenac formation was first detected 72 hours after treatment, its relative amount was even higher than the relative amount of free diclofenac. Production of the conjugate was found to increase over time and after 168 hours of diclofenac exposure it was the main metabolic product in plant leaves; representing 87% of the metabolic product (Fig. 3.43 ○).

The difference of the metabolic trend between root and leaf tissues is remarkable; while in roots the conjugation with sugar seems to be the characteristic pathway, in leaves OH-diclofenac is the main metabolic product. The fate of diclofenac-glucopyranoside in leaf tissues is undefined; it could be catabolized by further enzymatic cleavage. In that case, after cleavage of the glucosyl-moiety, the residual xenobiotic would be 4'-OH diclofenac.

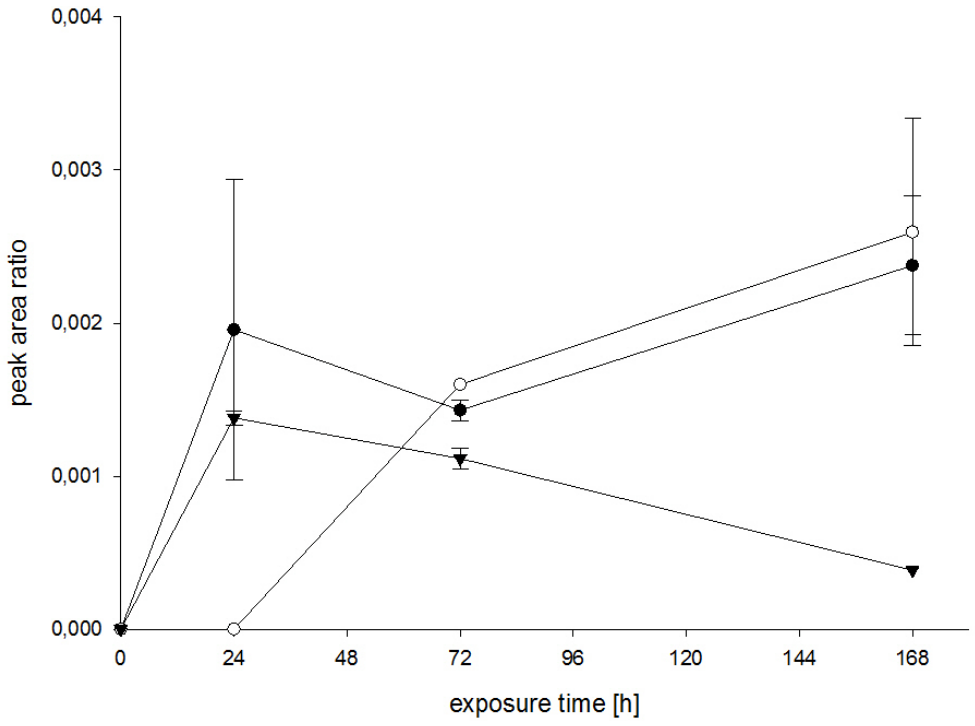


Figure 3.43 Relative amounts of diclofenac (●), 4'-OH diclofenac (○), and diclofenac-glucopyranoside (▼) conjugates in *Brassica* leaves after 1 mg/L pharmaceutical exposure. Error bars represent standard deviation of 3 biological replicates.

3.4.3 Effect of diclofenac on the Glycosyltransferase activity in *Brassica juncea*

The investigation of the time course of diclofenac metabolism in *B. juncea* refers to the important role of Glycosyltransferases during the detoxification process. The enzyme activity changes were determined in *Brassica* after 1 mg/L diclofenac exposure using the substrates TCP, quercetin and kaempferol. Table 3.6 depicts the GT activity changes in root and leaf tissues.

Although diclofenac-glucopyranoside is the main metabolite in *Brassica* roots, increase of Glycosyltransferase enzyme activity was not observed, neither with TCP, nor with the endogenous substrates quercetin and kaempferol. Enzyme activities were at the same level as in control plants, except for a slight increase after seven days of exposure, when using quercetin (123% of control) and kaempferol (132% of control). However, this had no statistical significance.

In leaf tissues even decreased activity of GT was detected using TCP; 24% of control activity after 24 hours of exposure, followed by a slight increase, but still under the enzyme activity level compared to control (50% of control after 3 days and 34% of control after 7 days of exposure). With quercetin and kaempferol as respective substrates a transient enzyme activity increase was observed. 24 hours diclofenac exposure led to decreased GT activity (about 30% of control). After 72 hours a slight increase was detected (136% and 130% of control). One week after treatment the enzyme activity was depleted and decreased again to 34% and 63% of control.

Table 3.6 Glycosyltransferase activity in *Brassica* control and 1 mg/L diclofenac treated plants. Changes in enzyme activity are also represented as control %. ± S.D. was calculated from three biological replication.

GT activity [pkat/mg protein]						
Time	Roots			Leaves		
	TCP			TCP		
	control	diclofenac	control %	control	paracetamol	control %
24 hr	3.4 ± 0.02	2.88 ± 0.49	93.4 %	9.07 ± 1.5	2.15 ± 0.44	24 %
72 hr	4.47 ± 0.17	1.0 ± 0.37	22.5 %	8.34 ± 0.2	4.2 ± 0.9	50.4 %
168 hr	3.7 ± 0.71	1.27 ± 0.05	33.9 %	8.16 ± 0.12	2.8 ± 0.02	34.5 %
	quercetin			quercetin		
	control	paracetamol	control %	control	paracetamol	control %
24 hr	6.96 ± 0.19	6.2 ± 0.15	89 %	28.1 ± 1.3	8.6 ± 1.07	31 %
72 hr	7.45 ± 1.6	6.89 ± 0.3	92 %	23.54 ± 0.7	32.08 ± 1.3	136 %
168 hr	5.44 ± 0.27	6.8 ± 0.6	123 %	27.68 ± 1.03	15.5 ± 1.7	52.6 %
	kaempferol			kaempferol		
	Control	paracetamol	Control %	Control	paracetamol	Control %
24 hr	5.75 ± 0.1	5.6 ± 0.5	98 %	23.86 ± 1.03	8.69 ± 0.9	36 %
72 hr	6.12 ± 1.3	6.3 ± 0.6	102 %	23.49 ± 0.02	30.05 ± 1.6	130 %
168 hr	4.54 ± 0.75	6.0 ± 0.6	132 %	24.33 ± 0.19	15.4 ± 1.0	63 %

3.4.4 Effect of diclofenac on the Glutathione S-transferase activity in *Brassica juncea*

Even though the investigation of the time course of diclofenac metabolism showed no significant conjugation with glutathione, GST may have an important role in defense responses caused by the pharmaceutical treatment that might be related to oxidative stress. To better characterize the role of GSTs, the enzyme activity was determined in cytosolic and microsomal extract of *Brassica* plants using CDNB, NBOC, fluorodifen and pNpa as substrates.

Cytosolic GST activity

After a first activity decrease (56% of control after 24 hours of exposure) GSTs in plant roots recovered its activity and one week after treatment showed increased activity (167% of control), when CDNB was used as substrate (Fig. 3.44 a). On the other hand no significant enzyme activity change was observed in the leaves (Fig. 3.44 b).

Using NBOC as substrate, a moderate but not highly significant GST activity decrease was observed in roots (Fig. 3.45 a). In leaves however, a strong activation was measured over time; 4-fold activity increase was detected already 24 hours after diclofenac treatment, followed by further increase, which reached its maximum at 870% of control after 72 hours of exposure. At the end of the experiment activity depletion was observed, yet GST was still 220% of control activity (Fig. 3.45 b).

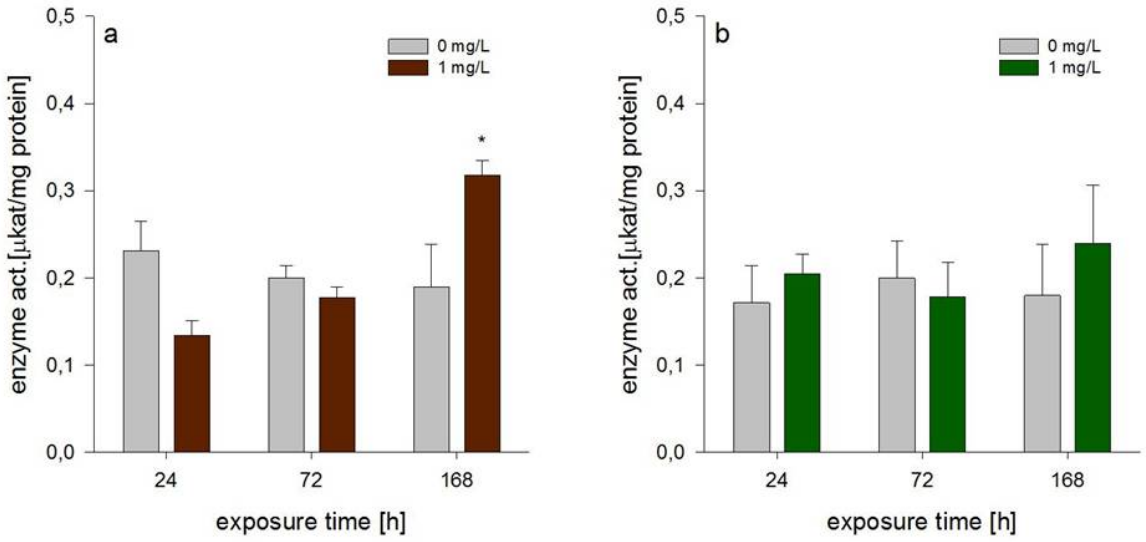


Figure 3.44 Cytosolic GST-CDNB activity in *Brassica* roots (a) and leaves (b) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05 vs. control.

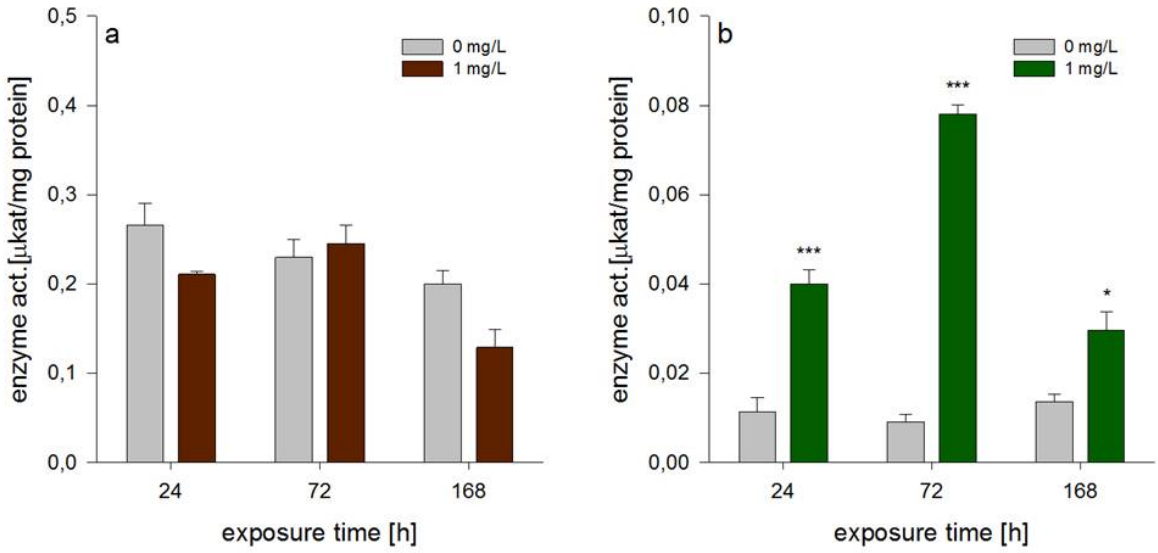


Figure 3.45 Cytosolic GST-NBOC activity in *Brassica* roots (a) and leaves (b) under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, ***p<0.001 vs. control.

Using the xenobiotic substrate fluorodifen, GST showed slightly decreased activity in root tissues after 24 hours (64% of control) and 72 hours (80% of control) of exposure. Activity returned to control level at the end of the experiment (Fig. 3.46 a). The observed enzyme activity changes were not significant in leaves, but in contrast to roots a trend towards activity increase could be observed (Fig. 3.46 b).

GST had elevated activity in plant roots for the conjugation of pNpa; 130% of control level was detected after 24 hours of exposure. This reached a maximum at 170% of control level after a slight recovery phase at the end of the one week treatment period (Fig. 3.46 c). The diclofenac treatment clearly effected GST activation in leaves. About 190% of control activity was detected after 24 and 72 hours, followed by further activity increase to 223% of control after 168 hours of exposure (Fig. 3.46 d).

Microsomal GST activity

GST activity was determined using CDNB, fluorodifen and pNpa as substrates, as in the experiments shown above. No microsomal GST activity was measured when NBOC was used as substrate.

Microsomal GST-CDNB activity was increased both in roots and leaves, however the increase in root tissues was moderate and first occurred after 72 hours of exposure (Fig. 3.47 a). In leaves the enzyme activity developed quickly; after 24 hours of exposure GST

activity increased to 290% of control. After that, activity depleted and returned to a slightly elevated level (150% of control) one week after treatment (Fig. 3.47 b).

In case of the fluorodifen substrate, the detected changes of GST activity were not strong (Fig. 3.47 c). In leaves a moderate activity increase was observed during the first three days of exposure; elevation to 131% of control after 24 hours and to 175% of control after 72 hours exposure time (Fig. 3.47 d).

After 24 hours of exposure a 2.7-fold GST activity increase was detected in roots using pNpa as substrate. Following this fast activation reaction the enzyme activity returned close to control level and showed no further changes till the end of the experiment (Fig. 3.47 d). Activity changes in the leaves of the treated plants showed no remarkable reaction during one week of diclofenac exposure (Fig. 3.47 f).

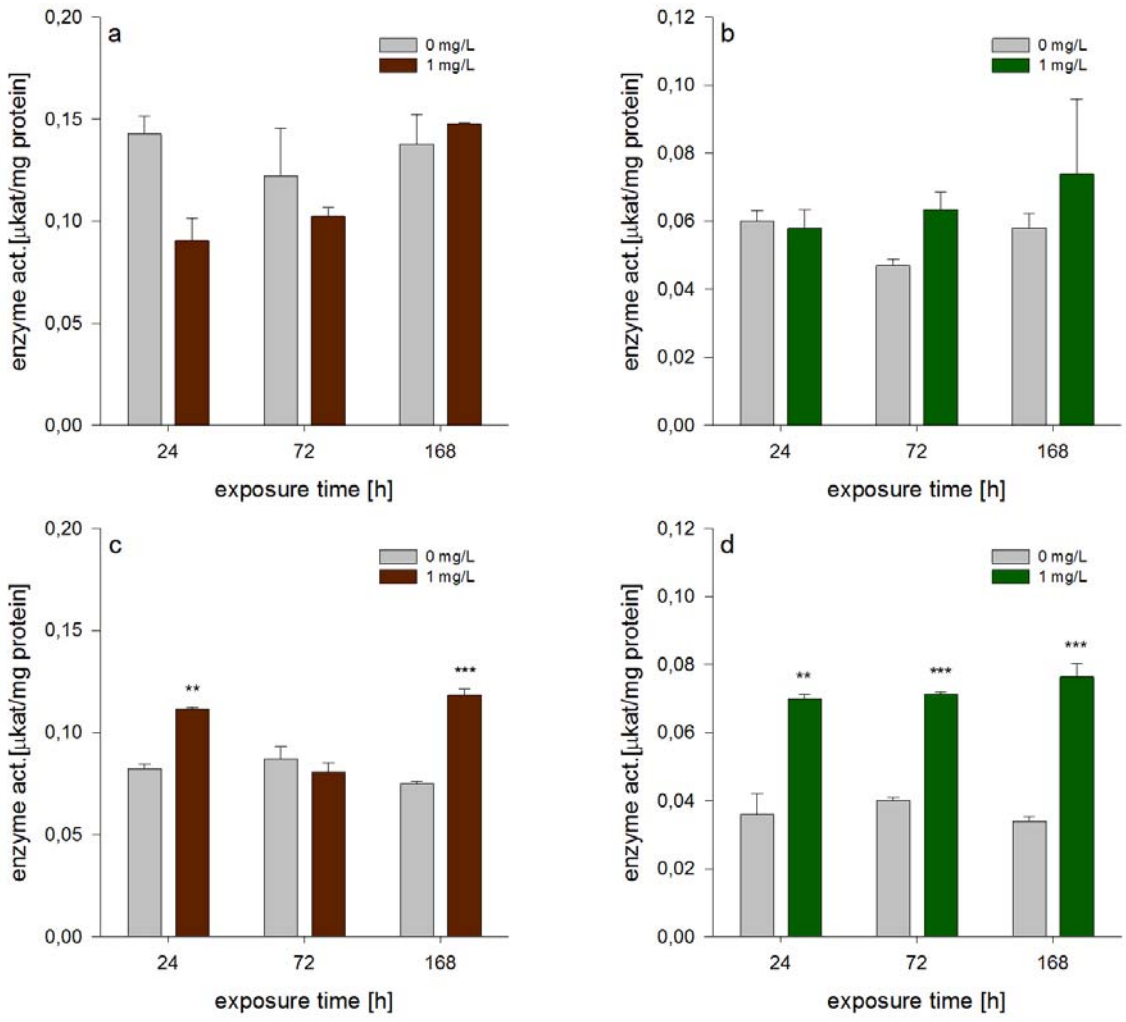


Figure 3.46 Cytosolic GST-fluorodifen activity in roots (a) and leaves (b), and GST-pNpa activity in roots (c) and leaves (d) of *Brassica* plants under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, **p<0.01 , ***p<0.001 vs. control.

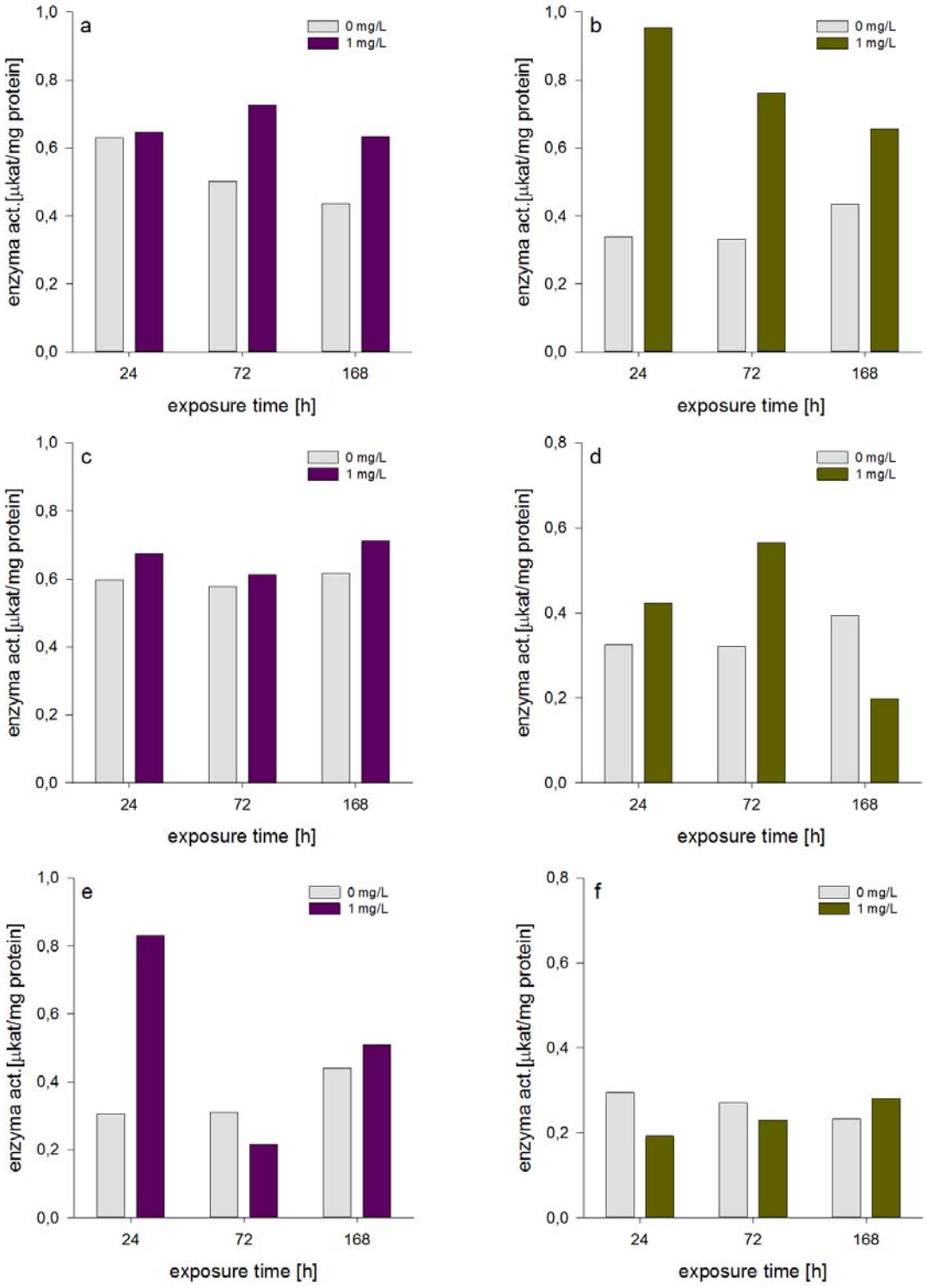


Figure 3.47 Microsomal GST activity in *Brassica* plants under 1 mg/L diclofenac exposure using different substrates to detect the enzyme activity. GST-CDNB activity in roots (a) and leaves (b), GST-fluorodifen activity in roots (c) and leaves (d) and GST-pNpa activity in roots (e) and leaves (f). Fresh biomass of three biological replicates was pooled during the extraction to reach the required fresh weight.

3.4.5 Specific GST gene induction in *Brassica juncea* under 1 mg/L diclofenac exposure

The selected Phi group GST, GST-F6, which has been frequently described as an oxidative stress marker in plants, showed strong up-regulation in plant roots during the first three days of diclofenac exposure. Contrary to the slower activation caused by paracetamol, diclofenac treatment led to a 7-fold relative gene induction already after 24 hours of exposure and, in spite of subsequent decrease, a 4-fold increased expression rate was detected after 72 hours of exposure (Fig. 3.48 a). Interestingly, in leaves the opposite effect was observed; GST-F6 gene expression was down-regulated during the whole experiment (Fig. 3.48 b), which indicates a moderate stress impact on the shoots of the plants.

GST-U5 showed a moderate up-regulation both in roots and leaves. In roots a 2.1-2.5-fold relative gene expression increase was observed after 24 and 72 hours of exposure. Thereafter the expression rate returned to the control level (Fig. 3.48 a), whereas in roots the same 2.6-fold increase was observed, but only after 72 hours of pharmaceutical exposure. At the end of the experiment the relative expression of GST-U5 in leaves decreased back to the control level too (Fig. 3.48 b).

The diclofenac treatment increased the expression of the other Tau group GST in the plant roots; 4.6-4.7-fold increase of GST-U24 gene expression was detected after 24 and 72 hours of exposure. As in case of the other GST genes, the up-regulation effect disappeared at the end of the experiment (Fig. 3.48 a). The gene was down-regulated in the leaf tissues during the whole exposure time (Fig. 3.48 b).

No significant changes were observed in GST-U19 gene regulation in roots, but in the shoot tissues the same down-regulation effect was observed as in case of GST-U24 and GST-U6 (Fig. 3.48).

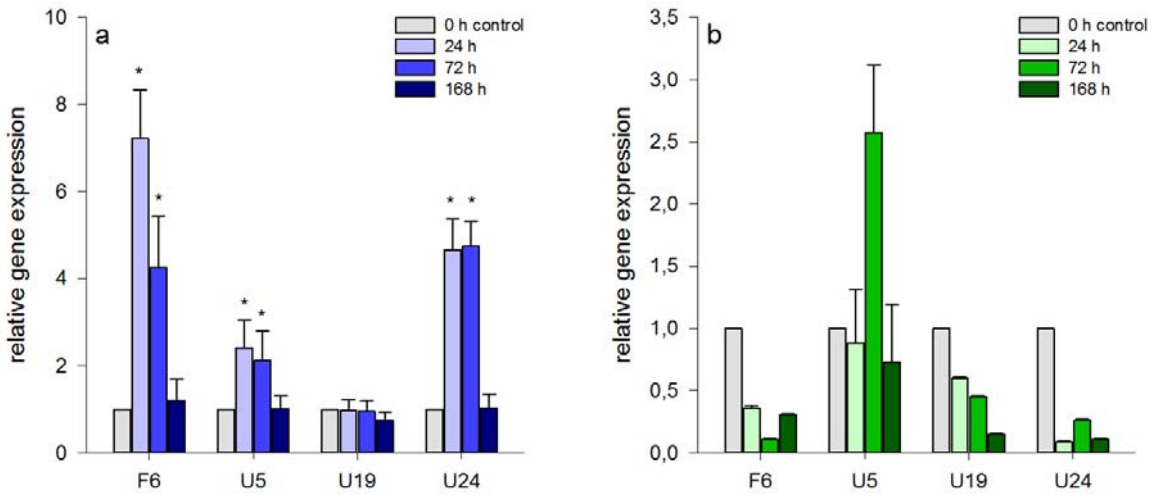


Figure 3.48 Relative gene expression changes of selected *Brassica* GST genes under 1 mg/L diclofenac exposure in roots (a) and leaves (b). GST expression levels were normalized to Actin and GAPDH housekeeping genes. Error bars represent ± S.D. of triplicate determinations.

3.4.6 Oxidative stress and enzymatic defense responses in *Brassica juncea*

To investigate the effect of diclofenac on the antioxidative defense enzymes, the activity of APOX and GR enzymes was measured in the cytosolic enzyme fraction. Furthermore POX enzyme activity was determined in cytosolic and microsomal extracts of *Brassica* plants exposed to 1 mg/L diclofenac.

Ascorbate peroxidase showed increased activity in roots already 24 hours after treatment at 260% of control. Subsequently enzyme activity was slightly above control level; 120% of control and 157% of control after 72 hours and 168 hours of exposure (fig. 3.49 a). Contrary to this, APOX showed decreased activity in the leaves; however changes were significant only after one day of exposure (30% of control) and seven days of exposure (56% of control) (Fig. 3.49 b).

After a fast activation effect in roots (270% of control 24 hours after treatment), GR showed depleted enzyme activity after 72 and 168 hours of exposure time (Fig. 3.49 c). More significant was the activity decrease in the plant leaves, where GR activity showed a 4-fold decrease 24 hours after treatment and maintained this low activity level for the remainder of the experiment (Fig. 3.49 d).

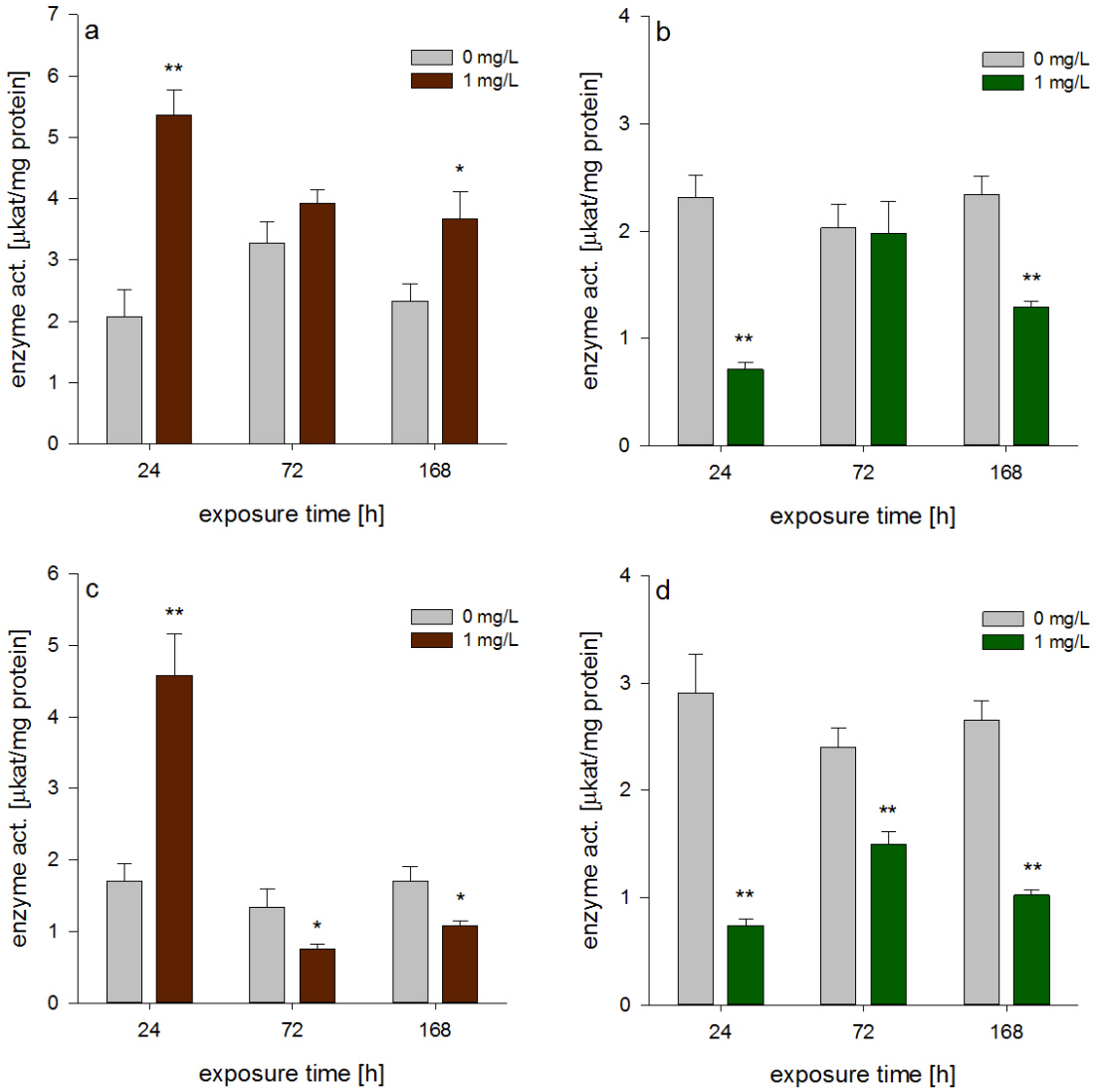


Figure 3.49 Specific enzyme activities in *Brassica* under 1 mg/L diclofenac exposure. APOX in roots (a) and leaves (b), GR in roots (c) and leaves (d). Error bars represent ± S.D. of 3 biological replicates. *p<0.05, **p<0.01 vs. control.

Cytosolic POX activity

Peroxidase activity showed time dependent activation in roots during the diclofenac exposure; the slight activity elevation 24 hours after treatment was followed by a 2.5-fold increase after 72 hours and reached its maximum one week after treatment with a 3-fold increased activity (Fig. 3.50 a). In leaves POX activity decreased over time after diclofenac exposure, although the relatively big standard deviation of three replicates (even in the control samples) does not allow a clear conclusion to be drawn (Fig. 3.50 b).

Microsomal POX activity

The opposite effect was observed in case of the microsomal POX activity during diclofenac exposure; enzyme activity decreased in roots to 45% of control after three days of exposure, but increased again close to control activity at the end of the experiment (Fig. 3.50 c). In leaves a clear activation of POX was detected during the whole experiment; enzyme activity increased up to 360-380% of control level and retained this level till the end of the experiment (Fig. 3. 50 d).

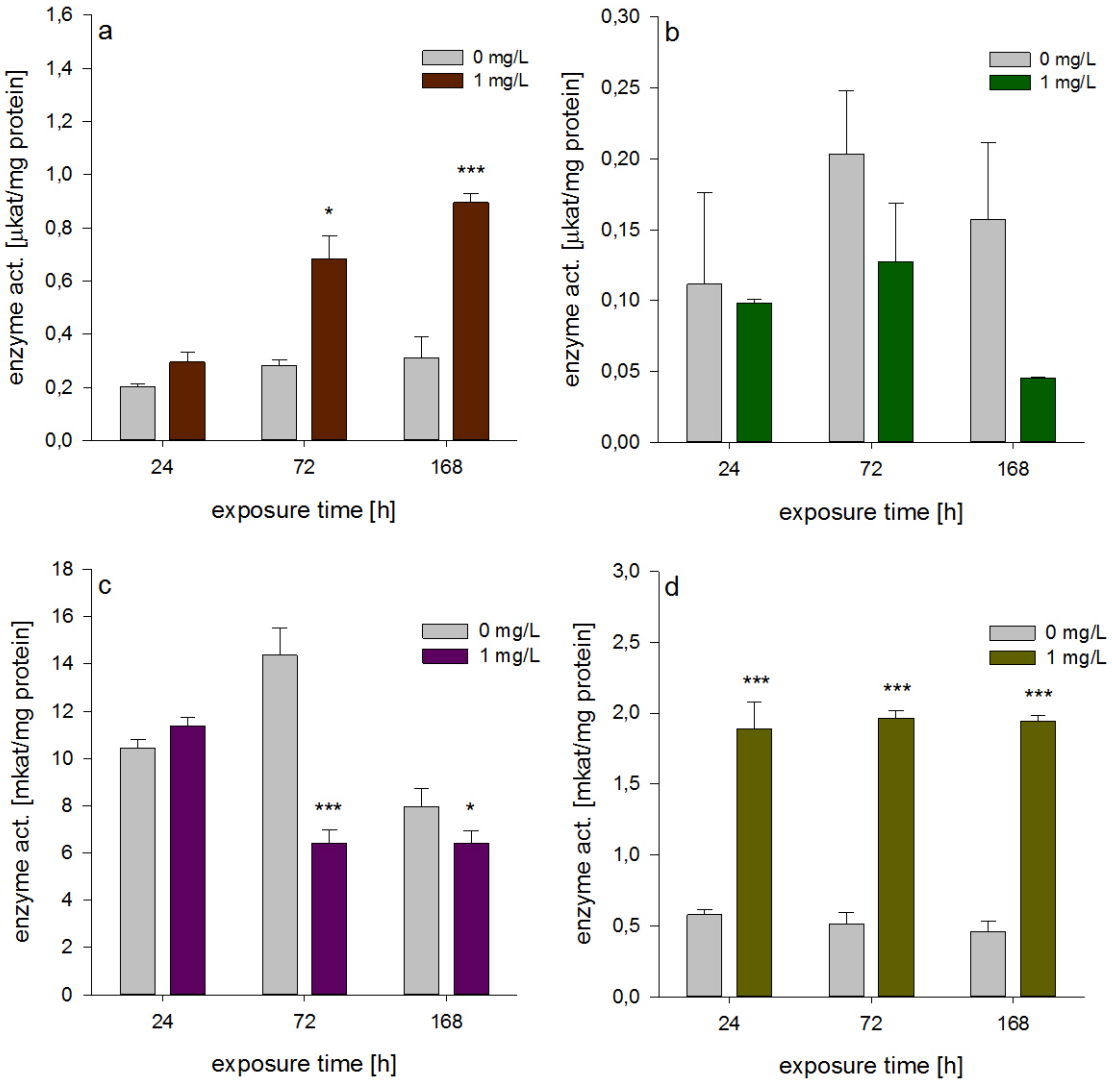


Figure 3.50 Cytosolic Peroxidase activity in roots (a) and shoots (b) and microsomal Peroxidase activity in roots (a) and leaves (b) of *Brassica* under 1 mg/L diclofenac exposure. Error bars represent ± S. D. of 3 biological replicates. *p<0.05, ***p<0.001 vs. control.

4 Discussion

In the last decade a „new generation" of pollutants raised growing concern in the field of environmental research: pharmaceuticals and personal care products, persistant chemical agents, which are used in huge amounts all over the world to make our life better, longer and healthier. Paradoxically, when these compounds enter our environment after use and due to their specific mechanisms of biological activity, they harm the affected ecosystems. In recent years pharmaceutical residues have been found not only in soils, air and waste, but even in the drinking water supplies of many countries. This highlights their possible impact on human health and the vicious circle connected to their unreflected use.

As the importance of the topic was growing in the last years; the European Union has established a Community framework for water protection and management with the main objectives of preventing and reducing pollution, promoting sustainable water usage, environmental protection, improving aquatic ecosystems and mitigating the effects of floods and droughts. Its ultimate objective is to achieve "good ecological and chemical status" for all Community waters (EU Water Framework Directive 2000).

Most drinking water contamination can be attributed to human activities. Examples for the negative impact of industrial chemicals are available in large numbers, but how drug residues affect the flora and fauna of rivers and lakes, has only been systematically evaluated in recent years. However, the question about the environmental consequences of drug use will become more prominent in the coming years, as more medicaments are needed in the future. As of now, risk and toxic effects of these agents are investigated in aquatic ecosystems, in order to establish ecotoxicological testing, mainly in invertebrates and fish, but the effects of pharmaceuticals on plants are much less investigated. This is a shortcoming, because plants might be able to act as living filters for such compounds, and can be used to phytoremediate polluted waters (Schröder et al. 2002). Therefore, the aim of this work was to investigate the pharmaca-plant interaction in two selected plant species, *Typha latifolia* L. (Common Cattail), and *Brassica juncea* L. Czern. (Indian mustard).

Typha is one of the most frequently used plants in constructed and semi-natural wastewater treatment systems. Many investigations point towards its great tolerance against toxic pollutants like heavy metals or organic xenobiotics and its capacity to remove them (Fediuc and Erdei 2001; Manios et al. 2002; Varun et al. 2011; Lyubenova and Schröder 2011). There is only one publication about pharmaceutical removal. Dorido et al.

(2008) reported about the efficient removal of the blood lipid regulator clofibric acid from water by *Typha*.

Brassica juncea is also frequently investigated as a heavy metal tolerant species, and widely used for the phytoextraction of toxic heavy metals from polluted soils (Clemente et al., 2005; Singh et al. 2005; Kumar et al. 1995). The tolerance of these plants to organic pollutants – especially to pharmaceuticals – has not been investigated so far.

The aim of this chapter is a critical analysis of the presented experimental results and their evaluation with respect to established scientific knowledge on this topic.

4.1 Uptake and translocation of human pharmaceuticals in plants

Our aim was to investigate human pharmaceutical uptake by plants from hydroponic culture medium, check for stress symptoms, and after that to investigate the distribution of metabolites in the plant tissues to explain the possible risk of entering these agents via plant accumulation into the food chain. Paracetamol and diclofenac were selected, two human painkillers consumed worldwide in huge amount, and frequently detected in reclaimed wastewater and natural water.

Contrary to removal, the physico-chemical basis for uptake of pharmacauticals has been studied in few papers; Kumar et al. (2005) found chlortetracycline uptake from soil in cabbage and green onion and transport to the arial part, with a 0.05-0.07% uptake ratio, Eggen et al. (2010) reported metformin, ciprofloxacin and narasin uptake from soil in barley and different cultivars of carrot, and transport to the upper part of plants in case of metformin. Carbamazepin, salbutamol, sulfamethoxazole and trimethoprim uptake from hydroponic culture into cabbage was reported by Herklotz et al. (2010), where also a higher concentration was described in roots than in aboveground tissues.

Investigations on the fate of paracetamol and diclofenac in plants are all based in our working group; short term uptake of paracetamol in common reed (*Phragmites australis*) rhizome tissue was described in the work of Neustifter (2007), where slices of the plant rhizomes were incubated in pharmaceutical solution. Similarly, Huber et al. (2009) reported paracetamol uptake in a hairy root culture of horseradish (*Armoracia rusticana* L.). Diclofenac uptake and distribution in barley was also described by Huber (2010).

In the present work uptake of paracetamol and diclofenac was demonstrated from liquid culture medium into the experimental plant species, *Typha* and *Brassica*. In both

cases, pharmaceuticals were already detected after 24 hours of exposure in the plant roots, followed by its transport to the arial parts of the plants.

The uptake rates in *Typha* (0.03% of paracetamol and 0.02% of diclofenac) were similar compared to literature data published by Kumar et al. (2005) for the case of veterinary antibiotics uptake by plants, or plant uptake of different human medicines like antibiotic, antiepileptic or bronchodilator agent described by Herklotz et al. (2010).

Brassica was found to have a ten-fold greater paracetamol uptake rate (0.3%), but only in case of exposure at high concentration. Plants exposed to low pharmaceutical concentration showed similar uptake rate like *Typha* plants; 0.01% in case of paracetamol and 0.07% in case of diclofenac.

Remarkable translocation of the pharmaceuticals to aboveground compartments was only observed in case of high paracetamol concentration treatments by *Brassica*; which transported a significant amount of paracetamol to the leaves (0.2% uptake rate). In the other cases the uptake rates in the plant shoots were minimum two decimal orders of magnitude lower (in range of 0.008-0.0001%). Of course, all these observations are based on young single plants under experimental greenhouse conditions. With view on the usefulness of the plants for phytoremediation purposes further studies under real life conditions would be required.

4.2 **Metabolisms of pharmaceuticals in *Brassica* and *Typha* plants**

Although the ability of plants to take up pharmaceuticals from their environment has been described several times in the literature (see above), their further metabolism in higher plants has not been investigated yet. Huber et al. (2009) published the first results in this topic, about the metabolism of paracetamol in the hairy root culture of horseradish. Further investigations were released also by Huber (2011) about diclofenac metabolism and metabolite identification in horseradish hairy root culture and in barley plants. This work further contributes to the understanding of the metabolic processes and the stress responses in higher plants caused by human painkillers.

4.2.1 Metabolism of paracetamol

Briefly, the main metabolic product of paracetamol in mammals is paracetamol glucuronide (40-60% of the conjugation products), followed by paracetamol sulphate conjugate (20-40%), and the paracetamol glutathione conjugate as a minor pathway (5-10%). In addition to the animal metabolism, fungal metabolism has been described, and the paracetamol glucoside conjugate was described by Huang et al., (2006) as the main metabolite in filamentous fungi. A summary of the metabolism of paracetamol in different organisms, including in the postulated metabolism in plants shown in Figure 4.1.

The first investigations of paracetamol metabolism in plants were published by Huber et al. (2009); interestingly, a branched metabolic pathway was described based on the metabolic products identified in suspension cultured horseradish hairy roots exposed to 1 mM paracetamol. The identified metabolites were on the one hand the paracetamol glucoside, but on the other hand, the paracetamol glutathione conjugate, and paracetamol cysteine conjugate, the further catabolic product of the glutathione conjugate were found.

According to our experiments we can conclude that the main metabolic product of paracetamol in both investigated plant species was the paracetamol glutathione. We base this conclusion to the experiments using low paracetamol concentration, where only the paracetamol glutathione product was clearly detected. Paracetamol conjugation with glucose was only observed in case of the 1 mM treatment, when paracetamol was present in toxic concentration. In this case, approximately the same amount of the two conjugation product was formed. In *Brassica*, the glucose conjugate even exceeded the amount of the glutathione metabolite.

These results show, that despite similarities to the human metabolism, other processes play a decisive role in the detoxification of pharmaceuticals in plants.

In the first step, an activation of paracetamol is necessary, which is known to be catalyzed by Cytochrom P450 monooxygenase enzymes in mammals. The resulting, highly active and toxic product, N-acetyl-p-benzoquinone imine (NAPQI) can only be detoxified when conjugated with GSH (Bertolini et al. 2006).

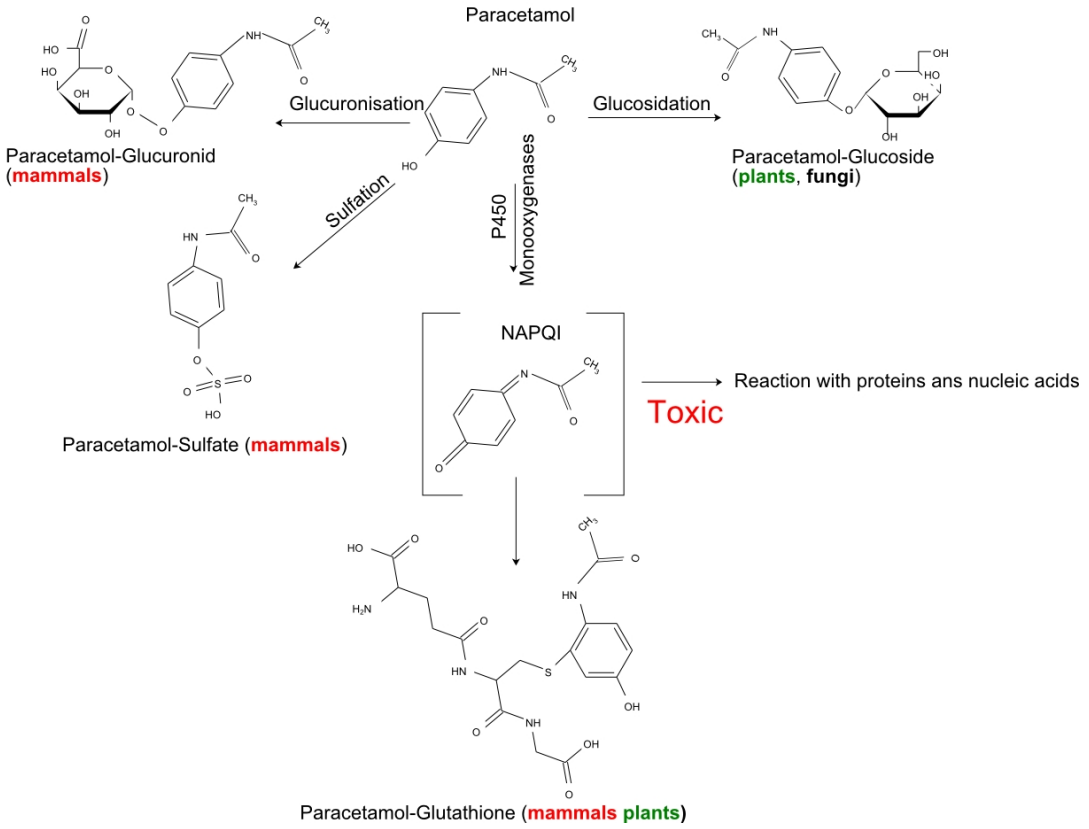


Figure 4.1 Metabolic products of paracetamol in mammals, plants and filamentous fungi (adapted from Huber, 2011).

Phase I metabolism of paracetamol

Cytochrom P450 monooxygenases are present also in higher plants, and play major role in the activation and detoxification of organic pollutants (Sandermann 1994; Werck-Reichart 2000; Kawahigashi 2007). Besides the P450 monooxygenases, Peroxidases are also able to oxidize and so activate xenobiotic compounds in plants. Stribova and coworkers (2000) described the activation of N-nitrosodimethylamine substrate both by P450 and Peroxidase enzymes. Moldeus and Rahimtula (1980) found an activation of paracetamol due to the peroxidase activity of prostaglandin synthetase in isolated sheep seminal vesicles, while Potter and Hinson (1987) detected the formation of GSH-paracetamol conjugates and paracetamol polymers catalyzed by Horseradish Peroxidase. These findings raise questions about the role of Peroxidases in the metabolism of xenobiotics, or specifically in this case in the metabolism of paracetamol in plants. An involvement of this enzyme class in the initial steps of paracetamol activation could be supported some factors; such as the much lower amounts of P450 Monooxygenases in plants than in animal tissues (Higashi 1988), and compared with this, the large amount of Peroxidases in plants, their inducibility, low

substrate specificity and their wide range of action (Stribova and Anzenbacher 1991; Jouili et al 2011).

Because the available methods in our laboratory did not allow to unambiguously detect the cytochrome P450 monooxygenase activity for paracetamol, it is not certain which enzyme has the key role in the Phase I metabolism of paracetamol in plants. The question is definitely worth for further investigation, because the role of Peroxidases during xenobiotic detoxification has not been frequently investigated yet.

Phase II metabolism of paracetamol

After the activation process, paracetamol will be conjugated with the tripeptide glutathione (GSH) in mammals, as in plants. In mammals this conjugation seems to be a spontaneous process, without GST enzyme participation, however different results can be found in literature about the topic. Jones et al. (1979) reported the role of γ-glutamyltransferase and cysteinylglycine dipeptidase, but not of GST in the paracetamol glutathione conjugation in isolated rat kidney epithelial cells, or Potter et al. (1985) described the *in vitro* formation of paracetamol-GSH conjugate in the presence of Horseradish Peroxidase, different concentrations of paracetamol, GSH and H_2O_2, without the presence of GST enzyme. However, when, Yonamine et al. (1996) reported about paracetamol derived activation of liver microsomal GST in rats, or Allameh and Alikhani (2002) described similar results, they found distinctly activated GSTs in response to paracetamol treatment in rat livers. Kozer et al. (2002) reported about increased GST enzyme activity in blood samples of paracetamol treated children, however, the authors were not sure that this effect was not triggered by the occurring oxidative stress.

Based on those literature data, as well as the well known role of the enzyme family in plants during xenobiotic detoxification we assume the important role of the enzyme in the conjugation process in plants. The role of the GST enzyme family during pharmaceutical detoxification in the plants will be discussed in details in the following subsection.

An important difference between mammalian and plant secondary metabolism is the involvement of glucoronide and sulfate conjugation (like in case of paracetamol metabolism) in animals, while in plants conjugation with UDP-glucose catalyzed by Glycosyltransferase enzymes is the main metabolic process (Sandermann 1994; Messner et al. 2003; Bowles et al. 2003). A number of xenobiotic compounds are described as substrates in glucosylation reactions, like 2,4,5-trichlorophenol (TCP) and 3,4-

dichloroaniline (DCA) in *Arabidopsis* (Brazier-Hicks et al. 2007), or pentachlorophenol, 4-nitrophenol, 3,5-dibromo-4-hydroxybenzoic acid described also in *Arabidopsis* by Messner et al. (2003).

Interestingly, our investigations showed, that in case of paracetamol the conjugation with glucose is only significant, if the substrate is available in large quantities. Paracetamol sulphate, the other metabolic product of paracetamol produced in higher amounts in mammals could not be detected in our investigations.

4.2.2 Metabolism of diclofenac

The metabolism of diclofenac has widely been studied, and metabolism of the drug was described in mammals, several fungi and microbes. The main phase I metabolite is 4'-hydroxy diclofenac and 5'-hydroxy diclofenac described in mammals (Leemann et al. 1993; Shen et al. 1999), fungi (Ibrahim and El-Feraly 1996) and bacteria (Webster et al. 1998; Osorio-Lozada et al. 2007). The 4'-OH metabolic product was also identified by Huber (2011) in barley plants.

Reactive metabolites of diclofenac can originate from further oxidation of the hydroxylated metabolites. These benzoquinone imine intermediates (1'4'-diclofenac quinine imine and 2,5-diclofenac quinone imine) lead to oxidative stress and liver injury (Bolesterli 2003). To avoid toxicity, the quinine imines undergo glutathione conjugation, where the end products are the non toxic 4'-OH diclofenac glutathione and 5'-OH diclofenac glutathione conjugates, identified in rats and in human liver microsomes (Tang et al. 1998; Yu et al. 2003).

The other metabolic pathway described leading to glutathione conjugates is the acyl glucuronidation of diclofenac, which leads to the formation of intermediate reactive diclofenac-acyl-glucuronide conjugates. Grillo et al. (2003) described the ability of the diclofenac glucuronide metabolite to transacylate GSH, and found the linear time-dependent formation of diclofenac-S-acyl-glutathione conjugate in rat bile.

Phase I metabolism of diclofenac

Our investigations showed fundamental similarities to diclofenac metabolism in other organism; 4'-OH diclofenac was identified as one of the main product in as *Typha*, as like in *Brassica* plants exposed to diclofenac. Both, in roots and leaves the rapid and efficient hydroxylation of diclofenac was observed, however, only in the shoot tissues, in presence

of very low diclofenac concentration, the OH-product was the main, or in case of *Typha* the only identified metabolite.

It should be noted that Peroxidases might have a crucial role during the diclofenac metabolism. Unfortumately this could not further be investigated in the present study. In humans, the Peroxidase-catalyzed oxidation of diclofenac leads to the formation of nitroxide or cationic radicals, which may play an important role in the diclofenac-induced liver injury. These diclofenac radicals, similar to the other reactive metabolic products can undergo glutathione conjugation. Similar as described for paracetamol, this might be spontaneous reactions without enzymatic catalysis (Boelsterli, 2003). As a matter of fact, Peroxidase catalyzed processes may also exert diclofenac induced oxidative stress in plants.

Phase II metabolism of diclofenac

Similar to the experiments carried out with paracetamol, we identified a glucoside conjugate of diclofenac, described also by Huber (2011) in barley plants and in horseradish cell cultures. Interestingly, in the plant roots where diclofenac concentration was much higher compared to leaves, the diclofenac glucopyranoside was identified as the main metabolic product; its amount was twice as high as 4'-OH diclofenac both in *Typha* and in *Brassica* plants.

During our experiments we found a new metabolic product of diclofenac in the investigated plant species, which had previously not been described in plants; after MS/MS analysis we identified the product as the 4'-OH diclofenac glutathione conjugate described by Tang et. al. (1998) in rats and in humans. We found the conjugate in very small quantity in plant roots; only 1% of the total conjugated diclofenac in *Brassica* and 2% in *Typha* was present in form of diclofenac glutathione metabolite.

The results point to a concentration-dependent activation of metabolic pathways in the plants; at low concentration the hydroxylation process seem to be sufficient to detoxify the xenobiotic compound, while at higher concentration new, perhaps more potent metabolic pathways are activated. A summary of the metabolism of diclofenac in different organisms, including in the postulated metabolism in plants shown in Figure 4.2.

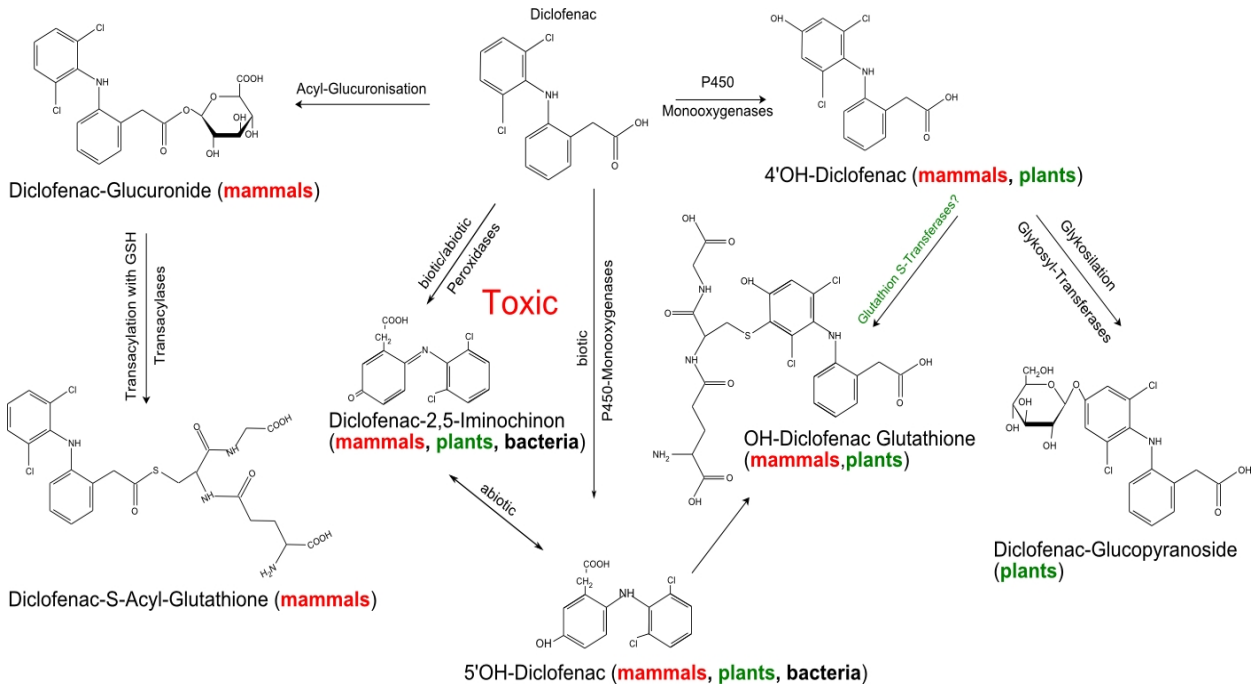


Figure 4.2 Metabolic products of diclofenac in mammals, plants and bacterial microorganisms (adapted from Huber, 2011).

4.3 **Pharmaceutical induced enzyme reactions in *Typha* and *Brassica* plants**

4.3.1 Glycosyltransferase activity

Glycosyltransferases transfer a sugar molecule from an activated sugar donor to an acceptor molecule and as such are involved in plant secondary metabolism. The enzymes are able to recognize a large diversity of substrates including hormones, secondary metabolites and xenobiotics such as pesticides and herbicides (Jones and Vogt 2001; Ross et al. 2001). During our experiment we determined the effect of paracetamol and diclofenac on GT activity in *Brassica* and *Typha* plants under 1 mg/L pharmaceutical exposure.

The enzyme reaction was fundamentally different in the two investigated plant species. In *Typha* plants a positive correlation was found between enzyme activity changes and the metabolism investigations (i.e. increased enzyme activity in the presence of glucopyranoside conjugates). In *Brassica* plants either paracetamol or diclofenac treatment did not increase enzyme activation despite presence of metabolites.

Another interesting result of our investigations is the difference in efficiency between the substrates we used to determine enzyme activity. Differences between treated

and control plants were much clearer using the endogenous substrates than using the model xenobiotic substrate TCP. The same effect was observed by Huber (2011) in horseradish roots under diclofenac exposure. Lim et al. (2001, 2002) reported that GTs, which usually glycosylate endogenous metabolites, recognize xenobiotics as substrate. Our results also confirm the hypothesis that individual Glycosyltransferases have multiple functions in the plant.

4.3.2 Glutathione S-transferases: function in pharmaceutical detoxification and stress response

GSTs are one of the most intensely investigated enzyme families in plants; their central function in the plant metabolism has been described in many publications. They are inducible by many different stress situations such as pathogen attack (Mauch and Dudler 1993), drought, high or low temperature (Anderson and Davis 2003; Gallé et al. 2009), heavy metal (Smith et al. 2004) or xenobiotic stress (Schröder and Pflugmacher 1996; Anderson and Davis 2003). GSTs are also thought to be involved in oxidative stress response mechanisms and in detoxification processes (reviewed by Edwards and Dixon, 2000).

Knowledge of the human metabolism of pharmaceuticals raises questions about the role of GST enzymes in the conjugation of these substrates with GSH. The literature data suggest that this process is spontaneous and does not require any specific enzyme reaction. The possibility of such a spontaneous reaction has been discussed on the basis of the concept of electrophilic force (Coleman et al. 1997, 2001). In some cases the reported GST reactions were induced by oxidative stress (Kozer et al. 2002; Allameh and Alikhani 2002). However, we must not forget that although plants and animals have partially the same detoxification enzymes and in both cases xenobiotics detoxification follows the three-phase model, the mechanisms behind the process are very different.

In our experiments GST activity was measured during pharmaceutical exposure using different substrates to determine the functions of different isoenzymes. For better characterization we investigated the gene expression of selected GST genes, whose response to oxidative stress or xenobiotics detoxification had been previously described.

The general observation of our investigations is the increasing GST activity in both plant species induced by paracetamol as well as diclofenac. A similar induction had been described by Neustifter (2007) in *Phragmites* roots and rhizomes after paracetamol and

diclofenac exposure using CDNB as substrate. However, in this case we had no opportunity to compare specific reactions. GST activity increase had also been reported by Huber (2011) in barley plants treated with diclofenac; the CDNB-GST activity changes over time were very similar to those obtained in *Brassica* plants. Yet NBOC detection showed greater activity increase in *Brassica*. The results in *Typha* plants showed more differences; GST activity increase is higher and faster than in *H. vulgare*.

Another interesting observation is that GST activity, detected with the different substrates, does not show the same reaction in the two investigated plant species under the same pharmaceutical treatment. If we compare the effects of different treatments on the same plant species, we can observe a similar behavior. This result demonstrates the basic difference between plant stress tolerance and detoxification mechanism. Otherwise, the analysis of the enzyme activity changes alone does not allow a clear allocation of the specific enzyme function to a conjugation or an antioxidative defense function.

To solve this problem, we investigated the effect of the pharmaceuticals on the expression of specific GST genes in *Brassica* plants. Our study clearly shows the specific effect of the drugs on different GST isoforms. Activation of the selected phi group GST gene (GST-F6) in both plants after paracetamol and diclofenac exposure points to the oxidative stress in plants induced by the treatments. An increased expression rate of this gene has been frequently described during oxidative stress in plants induced by a broad range of stress factors (Maleck et al. 2000; Wagner et al. 2002; Lieberherr et al. 2003). Another interesting effect of the treatments was the clear expression up-regulation of a tau group GST (GST-U5). In the current literature however, it is described as a constitutively expressed gene without any significant induction under different stress conditions such as herbicides, oxidative stress or pathogen attack (Wagner et al. 2001). This result points to the specific effect of the pharmaceuticals on the plant metabolism. The gene induction pattern correlates also positively with enzyme activities recorded. It was possible to assign to microsomal NBOC and fluorodifen GST activity in roots to GST-F6, but the GST-U5 in leaves remains unrepresented by a substrate.

4.3.3 Pharmaceutical induced oxidative stress in *Typha* and *Brassica* plants

Reactive oxygen species (ROS) are unavoidable products of the aerobic metabolism; they are generated in plants during normal metabolic processes like photosynthesis or respiration. The increased ROS production under various biotic and abiotic stress conditions is a well known phenomenon, described in many cases. To avoid damages and keep the cellular redox status in balance, ROS production is controlled by enzymatic and non enzymatic defense systems (reviewed by Arora et al. 2002).

We investigated the antioxidant enzymatic responses of the plants during the pharmaceutical exposure to characterize the toxic effect of these pollutants on the plants and describe the efficiency of plant defense mechanisms. We measured the activity of Ascorbate peroxidase (APOX), Glutathione reductase (GR) and Peroxidase (POX).

As a general response to the pharmaceutical treatments, POX activity was increased in *Typha* and *Brassica* roots as well as shoots. The same effect was described by Huber (2011) in barley plants treated with diclofenac. The only exception was observed in *Brassica* roots after treatment with 1 mM paracetamol, where the high concentration of the xenobiotic led to toxicity and irreversible root damages. Peroxidases are considered as biomarkers indicating biotic and abiotic stress (reviewed by Passardi et al. 2005). As we already discussed, in case of paracetamol and diclofenac, peroxidases may also play a role in Phase I activation processes during detoxification.

APOX and GR, two key enzymes involved in hydrogen peroxide elimination and the non enzymatic ROS scavenger ascorbic acid regeneration pathway (also known as Hallivell-Asada pathway), show different reactions in *Typha* and in *Brassica* plants. In *Typha* both APOX and GR showed increased activity after 1 mM and 1 mg/L treatments. In *Brassica* both treatments had a negative effect in the long term. This activity curb was very significant in case of paracetamol. Initial enzyme activation was observed after diclofenac treatment. Thereafter enzyme activation ceased. Under 1 mM paracetamol exposure *Brassica* plants also showed visual stress symptoms like tissue necrosis, root growth inhibition and generally irreversible damages, while *Typha* plants were not visibly affected and maintained a healthy appearance. All these enzymatic responses point to a higher tolerance of the *Typha* plants and so confirm the literature data about its capacity to remove pollutions.

4.3.4 Conclusions

The present study confirms the ability of higher plants to take up human medicine residues from the environment. We determined low uptake and transport rates to the aboveground part of the plants, which indicates a theoretically low contamination hazard of the food chain due to pharmaceutical accumulation in plant shoots. However, in practical phytoremediation plant density and root surface might compensate for it. In addition we should note that this investigation does not provide information on the effect of long-term exposure to these substances, which is more related to natural conditions.

Both investigated plants are described as tolerant species used in phytoremediation tecnologies; *Typha* has great ability to remove nutrients that get washed in excessive amounts to wastewater, while *Brassica* is frequently used to remediate heavy metal polluted sites. Though both plants have good stress tolerance capacity the same level of tolerance to the organic pollutants investigated here is not prove. An important observation is the varying capacity of the two plant species in terms of uptake and distribution of investigated xenobiotic compounds. We can confirm the formidable ability of *Typha latifolia* to deal with these compounds, just as described by Dorido et al. (2008), who reported about the great capacity of plant species to remove human pharmaceutical compounds from municipal wastewater. This issue is definitely worth further investigation.

We can draw some fundamental conclusions about the metabolism of these substances in plants. According to our experimental results, paracetamol and diclofenac degradation in plants proceeds following the three-phase model of xenobiotic detoxification. Despite similarities to the human metabolism, these results show that other processes play a decisive role in the detoxification of pharmaceuticals in plants. This was described by Sandermann (1994) as the so called “green liver” concept.

During our investigations we came upon some interesting questions, which cannot be answered in this work, but they are definitively worth further research. One of these is the better characterization of the function of plant Peroxidases during xenobiotic metabolism. Another is the comparison of plant Peroxidases function with Cytochrome P450 monooxygenases. The determination of the role of the different GST enzymes also requires further investigation; nonetheless our results show no evidence of GST involvement in conjugation during the pharmaceutical metabolism. A more detailed analysis of specific gene expressions and their complex evaluation in the context of enzyme activity determination using different substrates may help to find evidence of GST involvement in conjugation.

The growing number of publications on pharmaceuticals and their effect on our environment shows the importance of this topic. Further studies are needed under realistic conditions and in long term experiments. The present work is just a first step in assessing the impact of this new generation of pollutants and their evaluation in terms of ecotoxicological risk.

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