TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Ökologischen Landbau und Pflanzenbausysteme

Phytoremediation of water contaminated with Oxybenzone:

Implications for plant-mediated uptake and transformation of a widely used UV-filter

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Zusammenfassung

Wenn Körperpflegeprodukte (PCPs) in großer Menge unkontrolliert in die Umwelt gelangen, stellen sie eine Bedrohung für unsere aquatischen Systeme dar. Aufgrund ihrer hohen jährlichen Produktionsmengen und der umfangreichen Anwendung ist dies unter anderem der Fall für zahlreiche Sonnenschutzmittel, und ihr Vorkommen in allen Umweltkompartimenten hat Bedenken hinsichtlich ihrer möglichen Auswirkungen aufgeworfen. Bisher fehlt es an Informationen über die mögliche Entfernung und Umwandlung von organischen UV-Schutz-Wirkstoffen aus Oberflächenwasser durch Pflanzenfilter. In dieser Arbeit wurde Oxybenzon (OBZ) als Repräsentant einer ubiquitär verbreiteten Wirkstoffklasse ausgewählt, um die Rolle der Pflanze zur Sanierung von mit UV-Filtern verunreinigtem Wasser zu untersuchen. Die Arbeit umfasst vier Studien wie unten beschrieben.

I. Eine Wurzelhaarkultur (Hairy root culture) von *Armoracia rusticana* wurde als Modellsystem verwendet, um die Transformationskapazität von OBZ in der Phytoremediation zu untersuchen. 100 µM OBZ wurden in das Kulturmedium gegeben, nach 3 h Inkubation wurden die Wurzeln für 2, 4, 6 und 24 h in frisches Medium ohne OBZ überführt. Zwei Hauptmetaboliten wurden hier zum ersten Mal in einer Pflanze nachgewiesen und als Oxybenzon-Glucosid (OBZ-Glu) und Oxybenzon-(6-*O*-malonyl)-glucosid (OBZ-Mal-Glu) identifiziert.

II. Um die Toleranz von Makrophyten gegenüber OBZ zu beurteilen, wurden junge gesunde Triebe von *Cyperus alternifolius* abgeschnitten und sofort mit OBZ-Konzentrationen bis zu 500 µM versetzt. Messungen des Pflanzenwachstums, Quantifizierung der reaktiven Sauerstoffspezies sowie der Aktivitäten antioxidativer Enzyme zeigten, dass die Pflanze über längere Zeit eine Exposition gegenüber 50 µM OBZ ohne Schaden tolerieren konnte, eine Konzentration, die dem Zehnfachen der in der Umwelt gefundenen Konzentration entspricht.

III. Um die Aufnahme und Translokation von OBZ in höheren Pflanzen zu bestimmen, wurde ein hydroponisches Experiment mit *Cyperus alternifolius* im Gewächshaus durchgeführt. Basierend auf den früheren Toleranzergebnissen wurden Cyperus-Pflanzen mit 5, 25 und 50 µM OBZ für 1, 3 und 5 Tage versetzt. Die Aufnahme von OBZ aus dem wässrigen Medium überstieg 70%, OBZ wurde

kontinuierlich aufgenommen und die Akkumulation dieser Verbindung in *Cyperus* war am höchsten in Wurzeln, gefolgt von den Stielen und den Blättern. Effiziente Transformation von OBZ wurde in allen Geweben gefunden, wobei OBZ-Glu und OBZ-Mal-OBZ erneut als Hauptmetaboliten identifiziert werden konnten. Zusätzlich wurde ein weiterer Metabolit nachgewiesen und als 2,4-Dihydroxybenzophenon identifiziert. Dihydroxybenzophenon ist ein wichtiger Metabolit von OBZ in Tieren.

IV. Da die Wasser-Verschmutzung durch verschiedene Arten von UV-Filtern geschieht, wurde eine Exposition der oben genannten Meerrettich- Wurzelkultur mit einer Mischung von Schadstoffen, dem OBZ und einem weiteren typischen nicht-organischen Sonnenschutz-Wirkstoff, dem weit verbreiteten Nanomaterial TiO_2, für 2, 4, 7 d durchgeführt. Co-Exposition gegenüber TiO_2 beschleunigte die Aufnahme von OBZ durch Wurzeln. Zusätzlich wurden Toxizitätseffekte von TiO_2 und OBZ auf die Keimung von Gerste und Tomate getestet. Die Ergebnisse zeigten unerwarteterweise, dass TiO_2 den negativen Effekt von OBZ auf die Saatkeimungsrate und das Wachstum der Primärwurzel kompensieren kann. Ähnlich belegte der Wachstums-Test mit *Lemna minor* in Mikrotiterplatten, dass TiO_2 das Wachstum stimuliert und die Pflanze vor der schädlichen Wirkung von OBZ schützen kann.

Die vorliegenden Ergebnisse zeigten das enorme Potenzial von Pflanzen, OBZ zu tolerieren, es aus der Umwelt zu entfernen und zu metabolisieren. Mit der vorliegenden Arbeit ist es gelungen, zu einem Verständnis der Prozesse zu gelangen, die bei der Remediation von OBZ-belastetem Wasser eine Rolle spielen. Auch der weitgehend vernachlässigte Aspekt der multiplen Verschmutzung konnte zumindest angerissen werden. So stellt diese Arbeit wichtige Erkenntnisse und detaillierte Informationen über die Phytoremediation umweltrelevanter UV-Filter zur Verfügung.

Abstract

Water bodies act as the sink for many contaminants and thus the aquatic environment including surface, ground and marine water have become vulnerable to the effects of pollutants. Especially emerging contaminants such as personal care products (PCPs) have been found to pose a threat to the aquatic systems. Owing to the large annual production and extensive application, PCPs enter into the environment in a quasi-permanent manner, and their ubiquity has raised concerns about their impacts on the environment. In contrast to the other contaminants of the PPCP class such as pharmaceuticals which have been studied in detail, information about the removal and transformation of organic UV filters by plant mediated system is lacking. In this thesis, oxybenzone (OBZ) was selected as a model compound to investigate the role of plants for remediating water contaminated with UV filters. The thesis includes four studies as described below.

I. A hairy root culture of *Armoracia rusticana* was assigned as a model system to study the transformation capacity of OBZ in phyto-treatment. 100 µM of OBZ was added into the culture medium, after 3 h incubation the roots were transferred into fresh medium without OBZ for 2, 4, 6 and 24 h. Two major metabolites were detected for the first time *in planta*, LC-MS/MS fragmentation and in vitro hydrolysis & synthesis confirmed the formation of OBZ metabolites as oxybenzone-glucoside and oxybenzone-(6-*O*-malonyl)-glucoside.

II. To assess the tolerance of macrophyte to OBZ, a detached plant system of aquatic plant was created. Shoots of *Cyperus alternifolius* were spiked with OBZ concentrations up to 500 µM. Measurements of plant growth, reactive oxygen species as well as activities of antioxidative enzymes showed that plants were able to tolerate 50 µM of OBZ, which is ten-fold the concentration detected in the environment.

III. To determine the uptake and translocation of OBZ in higher plants, a hydroponic experiment with *Cyperus alternifolius* was established in the greenhouse. Based on the former stress tolerance results, *Cyperus* was spiked with 5, 25 and 50 µM OBZ for 1, 3 and 5 d. Depletion of spiked OBZ from the aqueous medium exceeded 70%, OBZ was taken up continuously and accumulation of this compound in *Cyperus* had the following order: rhizomes>stems>leaves. Transformation of OBZ was

found in all tissues, OBZ-Glu and OBZ-Mal-OBZ were again observed as the main metabolites. In addition, another metabolite was detected and identified as 2,4-dihydroxybenzophenone (DHB) which has been shown as a common metabolite of OBZ in animals.

IV. Being aware of the simultaneous contamination by different types of UV filters in surface waters, an exposure of horseradish hairy roots with a mixture of pollutants - OBZ together with a typical non-organic sunscreen TiO_2 - was conducted for 2, 4, 7 d. This study reveals the influence of the nanoparticle TiO_2 on the accumulation and transformation of OBZ in plant. Co-exposure to TiO_2 accelerated the uptake of OBZ by roots. When in additional experiments toxicity effects of TiO_2 and OBZ were tested by evaluating seed development of barley and tomato, results showed that TiO_2 may release the negative effect of OBZ on the seed germination rate and root elongation. Similarly, growth inhibition microplate tests with *Lemna minor* showed that TiO_2 stimulated frond growth and obviously protected plantlets from the harmful effect of OBZ.

The present results display the huge potential of plants to remove OBZ from water and transform it to various metabolites. The horseradish hairy root culture has been proven to act as an appropriate model to study the uptake and detoxification capacity of plants to cope with OBZ. *Cyperus alternifolius*, which shows efficient performance, is a promising candidate for treating this compound. The experiment with co-exposure to OBZ & nano-Ti deepened the understanding of remediation of OBZ under the situation of mixed pollutions. Thus the above findings provide significant insights in the environmental fate of sunscreens and offer detailed novel information about the plant performance for phytoremediation of environmentally critical UV-filters.

Chapter 1

1 Introduction

1.1 Emerging contaminants in the environment

Organic UV filters are common components among the personal care products (PCPs), and the active ingredient may account for up to 20% of the total content in several products. Consumption of UV filters increased with the increasing awareness of protecting skin from sunburn and fear of skin cancer. However, the increasing application of UV filters inevitably enhanced their entry into the environment (Fig. 1), where they are to date regarded as *pseudo-persistent* emerging contaminants due to their permanent input. Recreational activities which release UV filters during wash off from skin are a direct input of UV filters into the aquatic environment. Body care followed by incomplete removal of organic UV filters from municipal wastewater treatment plants (WWTPs) is an another principal source (Fent et al., 2010; Ma et al., 2016). WWTPs have been designed for eliminating solids, suspended particulates, nutrients, and dissolved biodegradable organic matter from sewage, but not for the removal of emerging contaminants. Hence, an increasing proportion of PCPs has been shown to leave WWTPs unmetabolized or poorly degraded (Miège et al., 2009). On the other hand, there is an increasing trend of using reclaimed wastewater for irrigation or groundwater recharge due to the water shortage in many countries around the world. As a consequence, organic UV filters have been detected frequently in various environmental matrices and aquatic organisms (Tsui et al., 2015). Thus removal of these emerging contaminants is urgently needed to ensure safe water reuse and to prevent adverse effects on the aquatic environment.

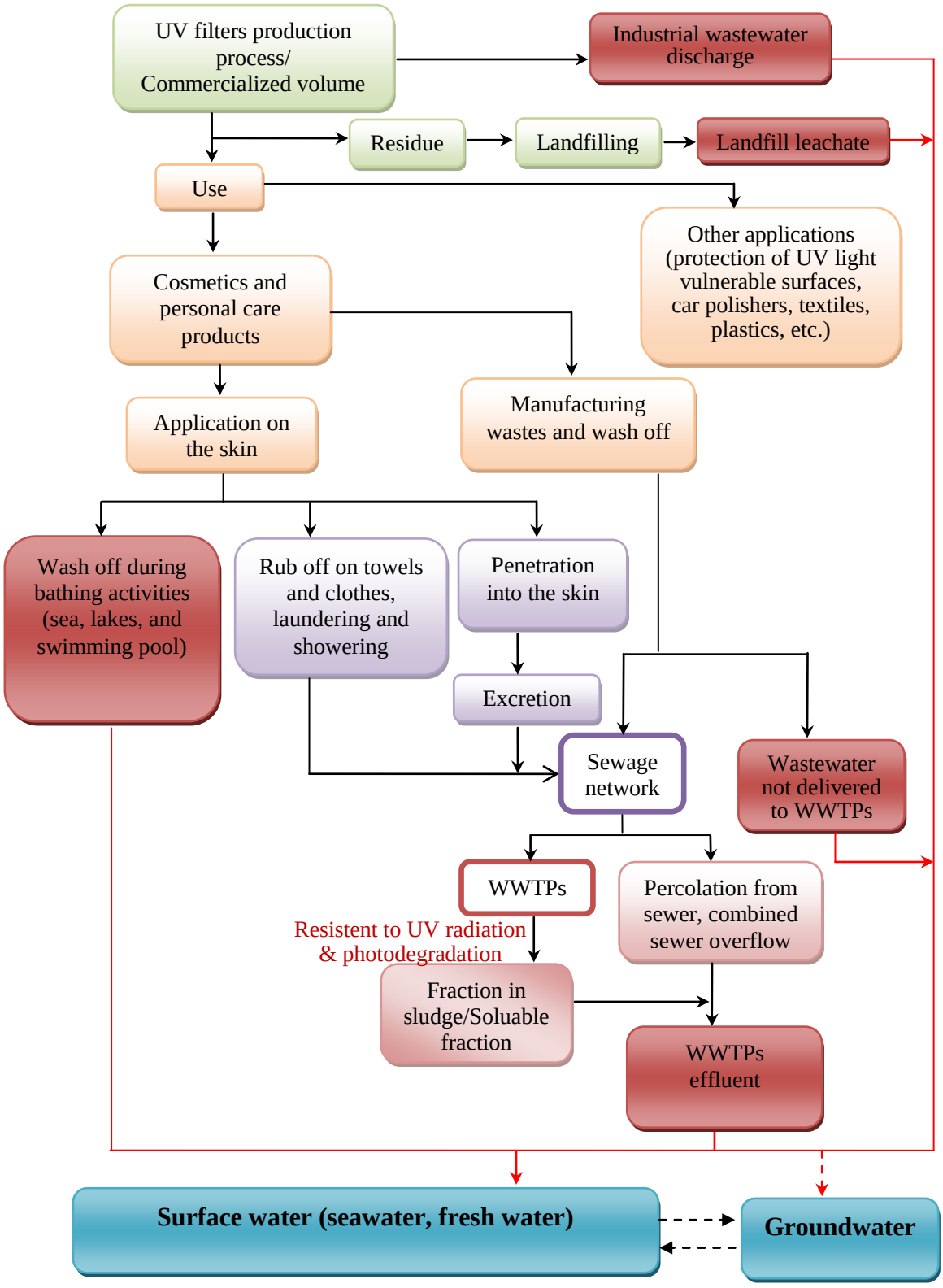


Figure 1 Pathway of UV filters in the environment. Boxes in red are inputs of UV filters, while the size of squares indicates the contribution of the respective inputs (adapted from Giokas et al., 2007).

1.2 The sun blocker Oxybenzone

1.2.1 Occurrence of OBZ in water bodies

Oxybenzone (OBZ) is an active ingredient in numerous PCPs, particularly skin lotions and makeup products. It has been available as a sunscreen agent for over 40 years (Kim and Choi, 2014). Widespread use of OBZ was documented in a United States National Health and Nutrition Examination Survey (Calafat et al., 2008), and over 81% of the 231 PCPs products collected from United States and China contain this compound (Liao and Kannan, 2014). In consequence, OBZ is one of the most frequently detected UV filters in surface water and wastewater and since 2005 it has been listed as an emerging contaminant due to its worldwide occurrence (Richardson and Ternes, 2014). In Europe, OBZ have been found consistently in Swiss rivers throughout May to August, without significant removal from WWTPs (Fent et al., 2010). High concentrations up to 3316 ng/L of OBZ have been detected in the Spanish islands (Rodríguez et al., 2015). In Asia, 722 ng/L and 5429 ng/L of OBZ was observed in secondary (activated sludge) treated wastewater and in surface seawater collected from coastal areas, respectively (Li et al., 2007; Tsui et al., 2014b). In America, the incredibly high concentration of 1.395 mg/L of OBZ was detected along Trunk Bay in Vrigin Islands. It is reported that 6000 - 14000 tons of sunscreen lotions which contain 1 - 10% OBZ are estimated to be released into coral reef areas annually, putting ca. 40% of coral reefs in coastal areas in danger (Downs et al., 2016). Surprisingly, OBZ occurred even in remote environments such as the Arctic Ocean as listed in Table 1 (Tsui et al., 2014b).

Table 1 List of OBZ concentrations in the environment

Aquatic environment	Concentrations (ng/L)	References
Oahu Island, Hawai'i	800 -1.92×10^4	(Downs et al., 2016)
Trunk Bay, U.S. Virgin Islands	0.6 × 10^6 - 1.4×10^6	(Downs et al., 2016)
Hawksnest Bay, U.S. Virgin Islands	7.5 × 104 - 9.5×10^4	(Downs et al., 2016)
Beaches and Los Angeles River, U.S.	227 - 601	(Tsui et al., 2014b)
Bay & river close to the WWTP discharge, New York	23 - 178	(Tsui et al., 2014b)
Surface seawater along beaches, Spain	1440 - 3300	(Tarazona et al., 2010)
Mogán beach, Gran Canaria, Spain	3316.7	(Rodríguez et al., 2015)
Influent of WWTPs, Switzerland	700 - 7800	(Balmer et al., 2005)
Effluent of WWTPs, Switzerland	10 - 700	(Balmer et al., 2005)
Hüttnersee, Switzerland	5 - 125	(Poiger et al., 2004)
Gray water from residential area, the Netherlands	300 - 4900	(Leal et al., 2010)
Influent WWTPs, Australia	2086 ± 1027	(Liu et al., 2012a)
Effluent of WWTPs, Australia	153 ± 121	(Liu et al., 2012a)
River, Bangkok, Thailand	86 - 116	(Tsui et al., 2014b)
Influent of WWTPs, Hongkong, China	284 - 557	(Tsui et al., 2014a)
Effluent of WWTPs, Hongkong, China	111 - 541	(Tsui et al., 2014a)
Harbor receiving 70% of WWTPs discharge, areas for aquatic recreational activities, Hong Kong, China	35 - 5429	(Tsui et al., 2014b)
Effluent of secondary treatment from WWTPs, China	97 - 148 (February) 538 - 722 (July) 292 - 343 (September)	(Li et al., 2007)
Arctic, arctic ocean and Chukchi Sea (65 and 75 °N)	17 - 33	(Tsui et al., 2014b)

1.2.2 Accumulation and effects of OBZ in biota

Recently, the bioaccumulation of OBZ in humans and other organisms has become a concern. OBZ has been detected in human breast milk and urine at concentrations up to 121.4 ng/g lipid and 5900 ng/mL, respectively (Kunisue et al., 2012; Schlumpf et al., 2008). The presence of OBZ in organisms involved in the food chain, particularly in fishes, increases the exposure of humans to this compound. Several studies have reported the accumulation of OBZ in aquatic species. Exposure to WWTPs effluents for 10 days resulted in a 325 ± 14 fold increase of OBZ-glucuronide concentration in bile of rainbow trout (*Oncorhynchus mykiss*) (Al-Salhi et al., 2012). 151 ng/g and 123 ng/g lipid weight of OBZ was detected in brown trout (*Salmo trutta*) and roach (*Rutilus rutilus*) from rivers and lakes, respectively (Balmer et al., 2005; Fent et al., 2010).

OBZ has been proven as an endocrine-active agent to fish and mammals. In adult zebrafish (*Danio rerio*) and eleuthero-embryos, OBZ concentrations in a range of 2.4 - 312 μg/L led to the down regulation of enzymes involved in steroidogenesis and hormonal pathways (Blüthgen et al., 2012). Significant induction of vitellogenin has been observed in OBZ treated juvenile rainbow trout (*Oncorhynchus mykiss*, at 747 μg/L OBZ) and male Japanese Medaka (*Oryzias latipes*, at 620 μg/L OBZ), and significant reductions in the egg production and hatching were detected at 16, 32 and 620 μg/L OBZ treatment (Coronado et al., 2008).

The deterioration of coral reefs has been associated with the presence of sunscreen UV filters including OBZ (Danovaro et al., 2008). An *in vitro* study has shown the deformation and DNA apyrimidinic lesions of coral planulae (*Stylophora pistillata*) after treatment with OBZ at 0.01 μM - 1 mM (2.28 μg/L - 228 mg/L). Exposure to OBZ led to the bleaching of planulae through decreasing of symbiotic dinoflagellate zooxanthellae and photosynthetic pigments, with the lowest observable effect concentration for bleaching being 2.28 μg/L and higher OBZ concentrations induced higher rate of coral bleaching (Downs et al., 2016). In addition, oxidative injury such as reduced glutathione contents has been shown in a protozoan ciliate (*Tetrahymena thermophile*) at 1 μg/L, and cell membranes impairment occurred after 4 h exposure to 10 mg/L OBZ (Gao et al., 2013).

OBZ concentrations detected in the wet season (May, August) are 30% higher than during the dry season (February, November), this seasonal pattern indicates a higher risk for aquatic organisms during their breeding season (Tsui et al., 2014a). According to the risk

quotients (RQ) calculated by dividing the measured environmental concentrations to the predicted no-effect concentration, OBZ posed highest risk potential due to its high RQ values (1.64) (Ma et al., 2016).

1.2.3 Current removal techniques for the OBZ

In light of the widespread occurrence and increasing concerns of organic UV filters as endocrine disruptors, there is a need for more effective removal, and the control of OBZ has been prioritized as the primary objective (Ma et al., 2016). OBZ is not supposed to be photodegradable due to its property as a sun-blocker, and the stability of OBZ towards UV irradiation was observed in previous reports of UV treatment of wastewaters (Liu et al., 2011; Tsui et al., 2015). Thus sorption onto sewage sludge and aerobic biodegradation are the main pathways for OBZ removal in WWTPs (Liu et al., 2012b). The reported treatments, preliminary, primary & secondary treatments, chlorination, UV disinfection, filtration over sand and activated carbon showed removal efficiencies of OBZ throughout the year in the range of 12 - 92% (Tsui et al., 2014a). A wastewater reclamation plant achieved removal efficiencies of 28 - 31% for OBZ along the treatments including coagulation–flocculation, continuous microfiltration and ozonation (Li et al., 2007). Treatment with reverse osmosis yields an effective removal efficiency of 99%, however, reverse osmosis is a comparatively expensive treatment method which has not yet been widely applied (Tsui et al., 2014a). Even at the removal rate of 92.7%, the predominant component of UV filters still present in the effluent is OBZ at concentrations of 153 ± 121 ng/L (Liu et al., 2012a). In this context, development of alternative treatment methods for effective elimination of OBZ is needed.

1.3 Phytoremediation

In recent years, phytotreatment has been proven as a potential approach to treat emerging micro-pollutants such as pharmaceuticals and personal care products (Matamoros and Bayona, 2006; Pilon-Smits, 2005; Schröder et al., 2007). Phytoremediation is the use of vegetation and its associated microorganisms to contain, extract or degrade contaminants (Green and Hoffnagle, 2004). It has been considered as a green & sustainable technology which requires lower energy input, low maintenance and has less destructive impacts than technical solutions. The major limitations of phytoremediation are generally time and area, because it takes longer time than other technologies and is susceptible to seasonal changes, and it also requires larger tracts of land. Other advantages and limitations of

phytoremediation are listed in Table 2 (Green and Hoffnagle, 2004; ITRC, 2009; Pilon-Smits, 2005).

Table 2 Additional advantages and limitations of phytoremediation

Advantages	**Limitations**
• *In situ* treatment • Improved aesthetics • Provide natural habitat • Wide public acceptance • Non-toxic final metabolites • Applicable to remote locations • Reduced generation of secondary waste • Controls erosion, runoff and dust emissions • Effective at low levels of mixed contamination • As ternary or polishing step attached to traditional approaches	• Limited root depth • Plant tolerance to pollutants • Unclear fate of contaminants • Disposal of pollutants in biomass • Potential transfer of pollutants via food chain

Constructed wetlands (CWs) are the applications of phytotechnologies to remediate water impacted with contaminants. While the dominant abiotic processes include sedimentation of particulates and suspended solids, adsorption to the sediments, precipitation by conversion to insoluble solids, photo-degradation and volatilization (Fig. 2a), the use of CWs, involves the biology of the selected hydrophytic vegetation. Biological removal of organic compounds includes plant uptake & translocation, degradation by microorganisms and plant enzymes, and tolerance mechanisms as shown in Fig. 2b (Green and Hoffnagle, 2004; Pilon-Smits, 2005), among these, direct uptake by plant is one of major contributors to the removal of contaminants. In general, organic xenobiotics with lipophilicity close to that of the plant roots tend to be taken up via diffusion. The octanol/water partition coefficient (log K_{ow}) is an important parameter to predict the uptake of xenobiotics by plants, contaminants with log $K_{ow} < 1$ are water soluble and will barely penetrate root epidermis, while compounds with log $K_{ow} > 3.5$ show high sorption to roots but low translocation to aboveground tissues, therefore xenobiotics with log K_{ow} between 1 and 3.5 are considered as optimal targets (Schwitzguébel et al., 2011). Subsequently, the contaminant can be metabolized through various enzymatic reactions and metabolic processes by plant enzymes, both in roots and shoots. In the next step, compounds are

stored and/or metabolized, or further carried over via transpiration stream to the aboveground tissues and finally sequestered there.

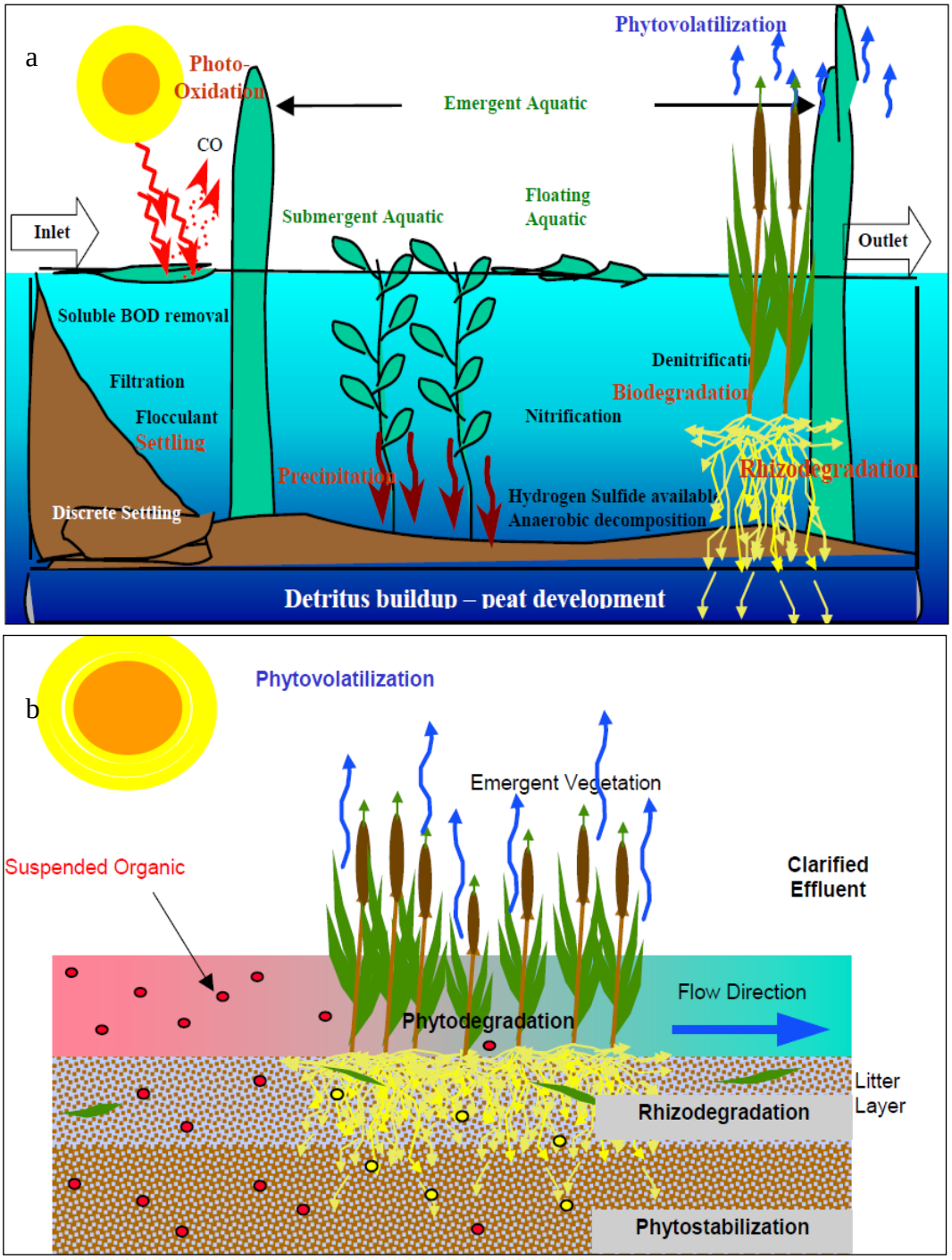


Figure 2 Main processes occurring in constructed wetlands. The removal mechanisms in CWs mainly involve abiotic (physical/chemical) or biotic (microbial/phytological) processes (a). Specifically an array of biotic processes contributes to removal of organic compounds in CWs (b) (ITRC, 2003).

CWs have been recommended as a supplement to conventional WWTPs, Fig. 3 shows a proposed combination of phytotreatment with a traditional existing sewage treatment to improve the removal efficiency. CWs may consist of two main types: surface flow (SF) and sub-surface flow (SSF) systems. SF wetlands have large surface area, and water surface moves above the substrate at low velocities in a quiescent manner (Fig. 4 a & b). In SSF wetlands, water flows below ground surface through the substrate (Fig. 4 c & d), SSF are known as gravel beds and vegetated submerged beds which are constructed with porous materials including soil, sand or gravel. SSF has the advantages of increased treatment efficiencies and more surface area for the bacterial biofilm growth over the SF wetlands. There are two types of SSF: horizontal flow (Fig. 4 c) and vertical flow (Fig. 4 d), horizontal flow system is more prevalent (ITRC, 2003; Scragg, 2005), for example, Fig. 5 shows a horizontal sub-surface flow system in Jiangchuan, China, this CWs is designed for treating domestic sewage in rural areas with a treatment capacity of 10000 m^3/d.

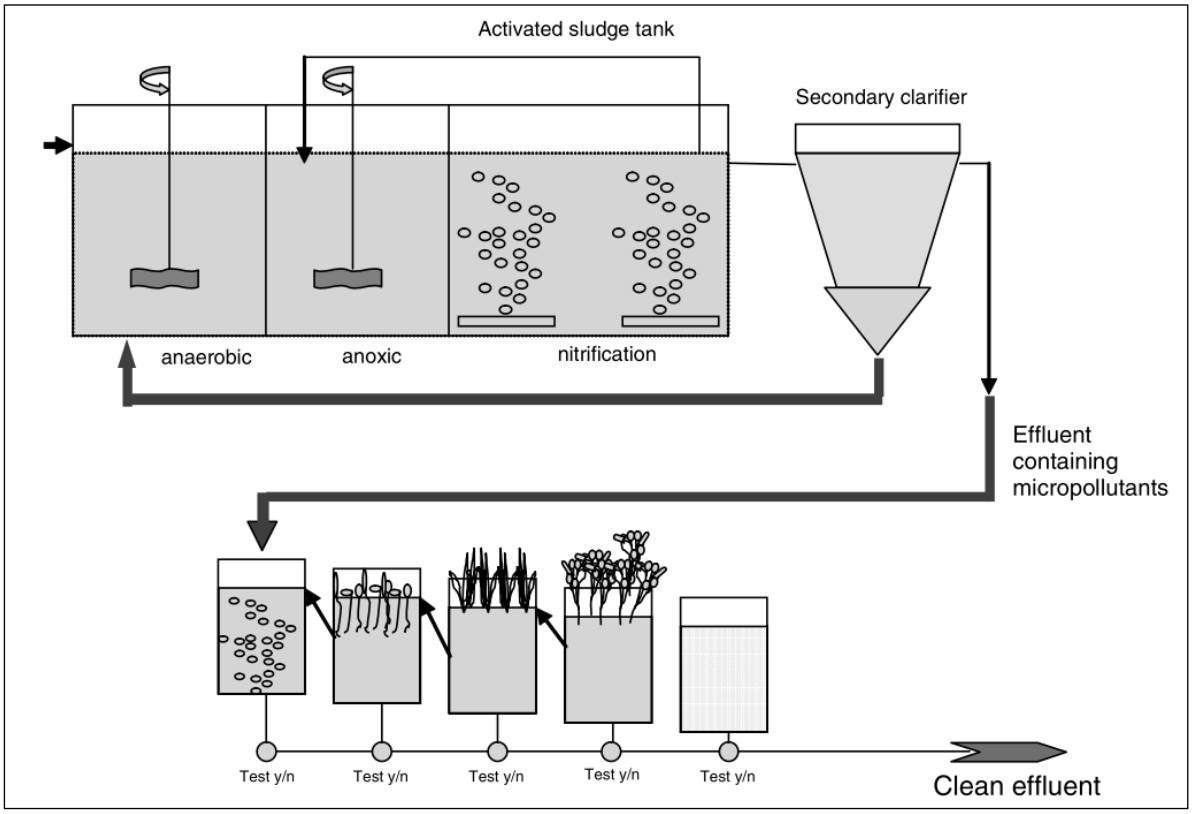


Figure 3 Sewage treatment facilities combined with optional phytoremediation modules (Schröder et al., 2007)

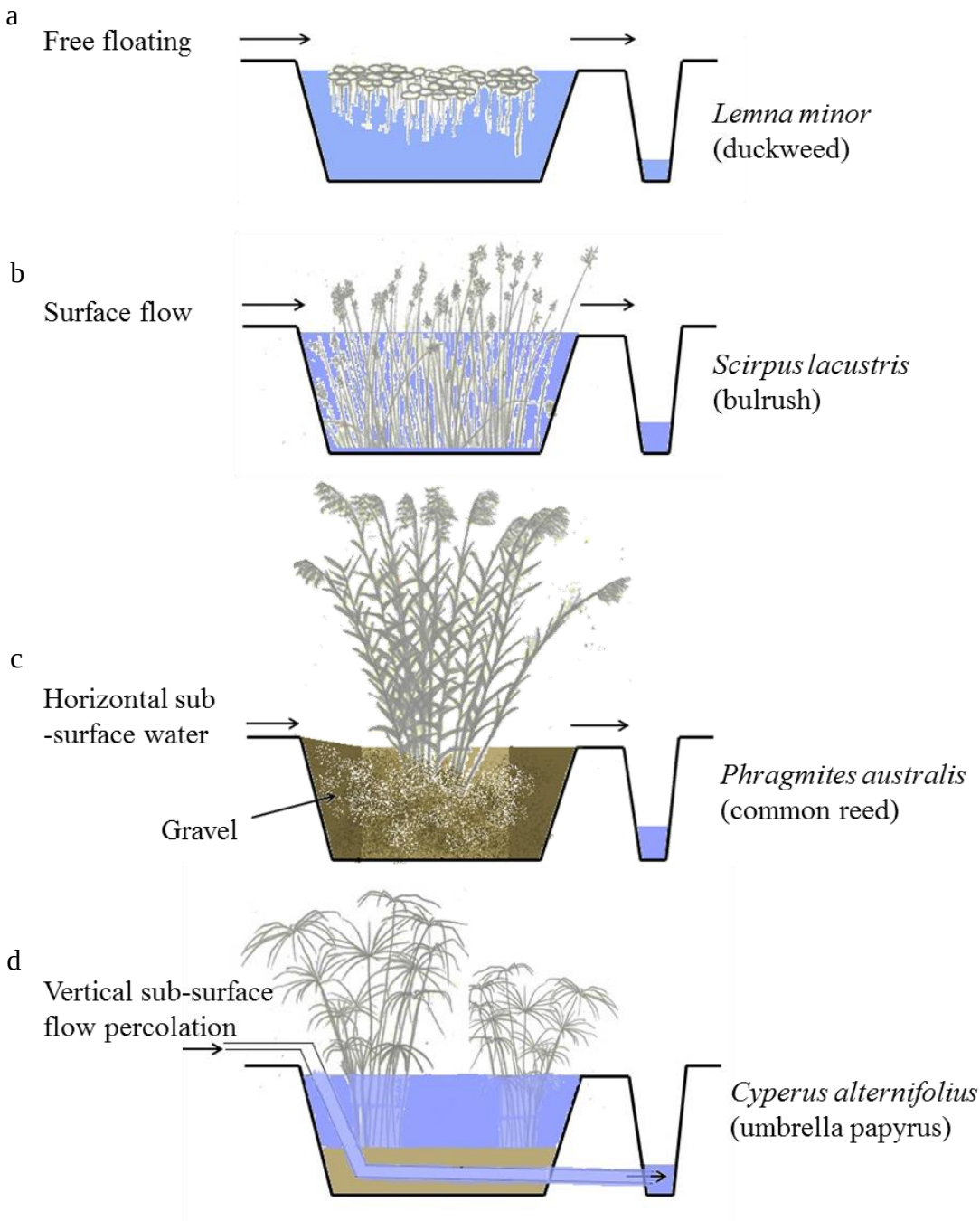


Figure 4 Different types of constructed wetland systems (adapted from Scragg, 2005).

Initial phase

3-year after implementation

Influent

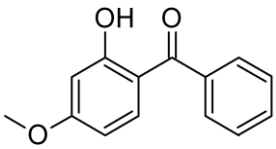
Effluent

Figure 5 Horizontal subsurface flow CWs (13000 m^2) in Jiangchuan, Yunnan Province, China (China Ecological Restoration, 2011)

1.4 Phytotreatment of OBZ: status and concerns

OBZ as a neutral compound with mild hydrophobicity (physiochemical properties listed in Table 3) is postulated to be removed by plant uptake (Matamoros et al., 2007). CWs have shown high efficiencies in removing OBZ. Ferreira et al. (2017) reported 96 - 97% removal of OBZ in the simulated CWs spiked with effluent collected after the secondary treatment in WWTPs. In a vertical subsurface flow constructed wetland, OBZ was nearly completely removed (95 - 97%) (Matamoros et al., 2007). However, in these studies only influent and effluent concentrations were measured to evaluate the performance of CWs, details about the final fate and removal pathways of OBZ are lacking. Also, to identify the potential applications of phytotechnologies, a decision tree such as shown in Fig. 6 is adopted. To aid this decision process for compounds like OBZ, typical set up for plant screening tests including laboratory, greenhouse hydroponics and potted plants are needed for answering the primary questions highlighted in the red frame in Fig. 6. Additionally, interactions between OBZ and other pollutants such as the nanoparticle TiO_2 needs to be considered in the co-contamination situation. As shown in Table 4, TiO_2 is frequently used as an active ingredient together with OBZ in sunscreen products, and its broad application as an inorganic UV filter as well as its occurrence in the water has become a concern in recent years (Kiser et al., 2009). Given the co-existence of OBZ and TiO_2 in the products, the likelihood of co-exposure to Ti-nanoparticles and OBZ is quite high; however, information about this area is inadequate.

Table 3 Physiochemical properties and chemical structure of OBZ

	Oxybenzone	Benzophenone-3
	CAS number	131-57-7
	Molecular formula	$C_{14}H_{12}O_3$
	Molecular weight	228.25
	Log K_{ow}	3.52

Source: pubchem

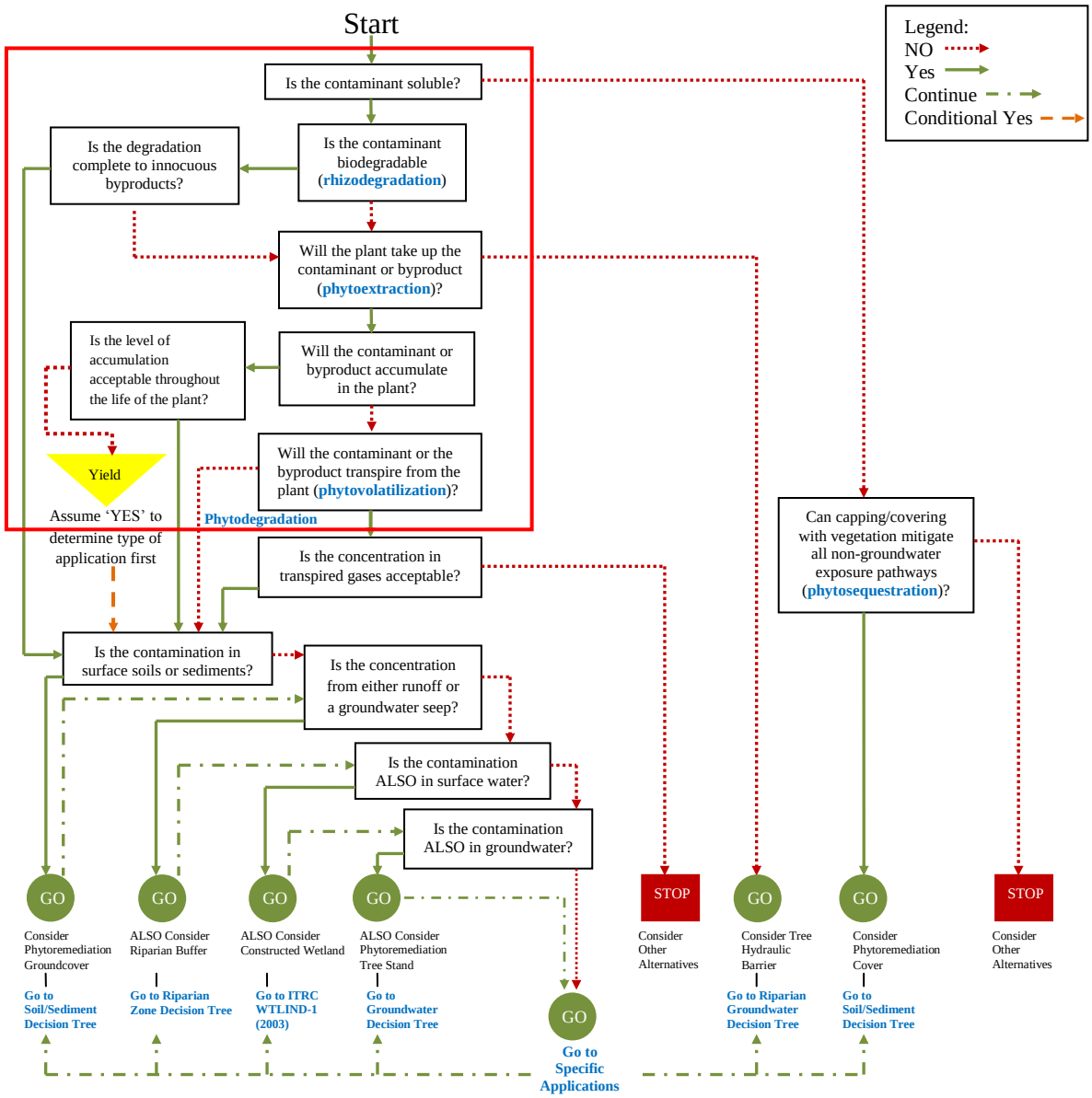


Figure 6 Remedy selection tree for phytoremediation (adapted from ITRC, 2009)

Table 4 Active ingredients in commercial sunscreen (Environmental Protection Agency, 2006; U.S. Food & Drug Administration, 2017)

Sunscreen ingredients	UV absorbance		Concentration in products	Chemical(C) or physical (P)
	UVA	UVB		
Oxybenzone	◐	●	6%	C
Titanium dioxide	◐	●	25%	P
Octocrylene	●	◔	10%	C
Zinc oxide	●	●	25%	P
Avobenzone	●	◔	3%	C
Protection level:	● extensive		◐ considerable	◔ limited

1.5 Aims of the thesis

To scrutinize the plant role in the fate and detoxification of OBZ in detail and to understand the interactions between contaminants under co-exposure, this thesis aims to investigate the capacity of phytotreatment of the representative UV filter OBZ in the hairy root model system and in a hydroponic system using a common macrophyte.

The main focuses of the thesis are as follows:

- To investigate the plant potential of transformation of OBZ in horseradish hairy root model system.
- To identify OBZ metabolites in plants.
- To evaluate plant stress response to OBZ treatment.
- To quantify uptake and translocation of OBZ in *Cyperus* hydroponic system.
- To determine OBZ toxicity, accumulation and transformation under mixed exposure with the nanoparticle TiO_2.

Chapter 2

2 Materials and methods

2.1 Plant material

2.1.1 Horseradish hairy root culture

Roots are the first organs to be in contact with contaminants. A hairy root (HR) culture is perfect system since it provides large surface area for contact between pollutants and roots in axenic conditions which exclude the interference of soil and microbes, thus it is considered as a valuable model in delineating the fundamental process for phytoremediation of organic and metal contaminants (Shanks and Morgan, 1999). The results obtained from HR can be extended to intact plants as the resulting metabolites in plants and plant cell cultures are in principle identical (Agostini et al., 2013). In this thesis, horseradish (*Armoracia rusticana* P. Gaertn., B. Mey. & Scherb., taxonomic classification in Table 5) was selected for studying the metabolism of xenobiotics due to its abundance of detoxification enzymes (Nepovím et al., 2004).

Hairy root culture of horseradish had formerly been obtained by transformation of nodal segments by *Agrobacterium rhizogenes* strain A4 (Nepovím et al., 2004). In short, sterilized horseradish seeds were germinated under axenic conditions on hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) at 27 °C and a light period of 18 hours. After the appearance of the second pair of leaves, nodal segments were cut and transferred onto fresh medium. Callus was produced when the wounded segments were inoculated with a suspension of *Agrobacterium rhizogenes* strain A4 (10^7 cells/mL) for 24 h. The newly transformed roots were excised and first cultured on MS medium supplied with phytohormones naphthalene acetic acid (NAA, 0.6 μM) and 6-benzylaminopurine (BAP, 4.4 μM). 500 mg/L ticarcillin was applied for three sub-cultivation

periods to eliminate bacteria. The MS medium used in the second and third sub-cultivation period was hormone free.

After transformation, the roots were grown in 250 mL Erlenmeyer flasks containing 100 mL MS medium with addition of 3% sucrose, 0.1g/L inositol and 0.32 mg/L thiamine (Table 6). The cultures were grown in the darkness on a shaker at room temperature. Sub-division was carried out every two weeks under sterile conditions; normally one culture was subdivided into four new cultures and transferred to the fresh medium.

Table 5 Taxonomic classification of *Armoracia rusticana*

Kingdom	Plantae
Division	Angiosperms
Class	Eudicots
Subclass	Rosids
Order	Brassicales
Family	Brassicaceae
Genus	*Armoracia*

Table 6 Composition of MS medium for horseradish hairy root cultures

Macroelements		**Microelements**	
$CaCl_2$	2.99 mM	$CoCl_2 \cdot 6H_2O$	0.11 μM
KH_2PO_4	1.25 mM	$CuSO_4 \cdot H_2O$	0.1 μM
KNO_3	18.79 mM	FeNa-EDTA	0.1 μM
$MgSO_4$	1.5 mM	H_3BO_3	0.1 μM
NH_4NO_3	20.61 mM	KI	5 μM
		$MnSO_4 \cdot 4H_2O$	0.1 μM
		$Na_2MoO_4 \cdot H_2O$	1.03 μM
		$ZnSO_4 \cdot 7H_2O$	29.91 μM

2.1.2 *Cyperus alternifolius* L.

Cyperus alternifolius, known as umbrella sedge, is perennial plant which belongs to the sedge family of Cyperaceae (taxonomic classification in Table 7). It originates from Madagascar and is widely naturalized in the tropical and subtropical areas (Baijnath, 1975). *Cyperus* is well suited as an ornamental plant cultivated in water gardens and as pot plant (Davenport et al., 1992). It is fast growing and can reach a height of 1.5 meters with strong underground rhizomes and roots, also it can be easily propagated by seeds or pieces of the plant (Ebrahimi et al., 2013).

Cyperus spp. has been widely used in many constructed wetlands for sewage wastewater and landfill leachate treatment (Hadad et al., 2006; Vymazal, 2013), Fig. 5 shows efficient treatment in SSF wetlands planted with *Cyperus*. This species tolerates harsh manipulation and adapts to hyper eutrophic and salinity conditions (Chan et al., 2008; Tao et al., 2015). Additionally, its nature of growing best in the tropical environment makes it an appropriate plant for removing OBZ, as high environmental OBZ concentrations were detected in tropical areas.

Seeds of *Cyperus* were purchased from a local provider (Tropica GmbH & Co KG, Münster, Germany) and sowed on wet tissues shielded from direct light. After germination, seedlings were transferred to soil and grown under the greenhouse conditions at 20/25 °C with light/dark cycle of 12/12 h and an average humidity of 65%. When the plants reached a height of 30 cm, they were isolated from soil and acclimatized to hydroponic growth conditions for four weeks before the experiments were performed in modified Hoagland nutrient solution (Table 8) (Dordio et al., 2009). The nutrient solution was replaced weekly.

Table 7 Taxonomic classification of *Cyperus alternifolius*

Kingdom	Plantae
Division	Angiosperms
Class	Monocotyledonae
Subclass	Commelinids
Order	Cyperales
Family	Cyperaceae
Genus	*Cyperus*

Table 8 Composition of Hoagland solution for *Cyperus alternifolius*

Macroelements		**Microelements**	
$MgSO_4 \cdot 7H_2O$	2 mM	H_3BO_3	10 μM
$Ca(NO_3)_2 \cdot 4H_2O$	2 mM	FeNa-EDTA	10 μM
KNO_3	2 mM	$MnCl_2 \cdot 4H_2O$	1 μM
KH_2PO_4	0.5 mM	$ZnSO_4 \cdot 7H_2O$	0.5 μM
$CuSO_4 \cdot 5H_2O$	0.5 μM		
$Na_2MoO_4 \cdot H_2O$	0.1 μM		

2.2 Experimental set up

2.2.1 Hairy root (HR) culture experiment

This experiment was designed to study the metabolism of OBZ in plants. Roots obtained from the same generation were adopted and grown in full strength MS medium for 10 days. 15 flasks of roots were spiked with 100 μM OBZ. After 3 h incubation, roots were washed with sterile water and transferred to fresh MS medium without OBZ. Triplicate roots were harvested at 0, 2, 4, 6 and 24 h after the transfer. Samples were dried with lint paper and frozen in liquid nitrogen and stored in -20 °C. All chemicals used were analytical grade.

2.2.2 Stress response experiment

Cyperus shoots with similar size were cut from the main plants; bundles of four young shoots with fully developed leaf whirls were immediately soaked in one falcon tube. Shoots were subjected to three concentrations (50, 100 and 500 μM) of OBZ, and plants free of OBZ treatment were considered as control. Samples were collected after 2, 4 and 7 days. Triplicate tubes were established for each OBZ concentration and each exposure time. After measuring the fresh weight, samples were frozen and stored at -80°C.

2.2.3 Hydroponic experiment with OBZ

Complete plants of *C. alternifolius* were placed into glass vessels containing Hoagland medium spiked with OBZ at 5, 25 and 50 μM, respectively. Plants without OBZ treatment were set up in parallel. The experiment was conducted in the greenhouse under the conditions mentioned above. Three replicate vessels were carried out for each concentration at each exposure period (1, 3, 5 days). The entire plants from triplicates were

harvested for different treatments at designated times, and rhizome, stem and leaf samples were carefully separated and frozen at -80°C for further analysis. To determine the OBZ concentration in the medium, 1 mL nutrient solutions were collected from each vessel and analyzed by HPLC.

2.2.4 Multiple-exposure experiment with OBZ and TiO_2 nanoparticles

The experiment was initiated by incubating horseradish hairy roots in the medium with the following four treatments: 1) 5 µM OBZ; 2) 3 mg/L TiO_2; 3) 5 µM OBZ + 3 mg/L TiO_2; 4) control free of OBZ and TiO_2, respectively. Medium without roots was set up under the same conditions. Each treatment contains triplicate samples. Roots and medium from all the treatments were harvested at 0, 2, 4 and 7 days, respectively. Root samples were frozen in liquid nitrogen and stored at -80 °C for further analysis.

The toxicity test was conducted with the floating plant *Lemna minor* in 96-well plates, where one frond was placed in one well. 24 wells were integrated for each treatment. Treatments including OBZ/TiO_2 alone or in mixture were set up similar to those described above. The growth inhibition was defined by the change of frond area after three-day incubation.

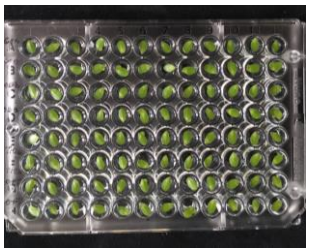
HR culture experiment	Stress response experiment	Hydroponic experiment	Toxicity test with *Lemna minor*
			

Figure 7 Experimental set up

2.3 Measurements

2.3.1 Solid phase extraction (SPE) of OBZ & its metabolites

OBZ and its metabolites were extracted with 1.5 mL H_2O/acetonitrile (30/70, v/v) from 0.5 g of ground plant material. After 5 min vortexing and 5 min ultra-sonication, samples were centrifuged at 13,000×g for 30 min. Supernatants were collected and then filtrated (0.45 μm, PVDF). Oasis HLB solid phase extraction columns (3 cm^3, 60 mg) were used for purifying samples. Cartridges were conditioned with 3 mL methanol and 3 mL water. 0.5 mL samples were loaded and the impurities were flushed by 3 mL water. After drying under vacuum, the analytes were eluted with 2 mL acetonitrile. To detect OBZ in the growth medium, PVDF syringe filters (0.45 μm) were used to remove impurities.

2.3.2 High performance liquid chromatography (HPLC) analysis

OBZ concentration in the nutrient medium was determined via HPLC (Varian ProStar 210, Germnay). Mobile phases consisted of ultrapure water (MilliQ, Millipore Corporation) with 0.1% trifluoroacetic acid as A and acetonitrile (HPLC grade) with 0.1% trifluoroacetic acid (Carl Roth, Germany) as B. Samples were separated on an Agilent eclipse XDB-C18 reversed-phase column (5 mm, 4.6 × 150 mm) with the following gradient: 0 - 1 min, 50% A (isocratic); 1 - 5 min, 90% B (linear increasing); 5 - 6 min, 50% A (linear decreasing); 6 - 7 min, 50% A (isocratic), flow rate maintained at 1.2 mL/min. OBZ was detected by an UV detector at 289 nm (Varian ProStar 335, Germany).

2.3.3 Liquid chromatography tandem mass spectrometry (LC-MS) analysis

Plant extracts samples were measured on an HPLC system (Varian ProStar 210) coupled to an ion trap mass spectrometer (Varian 500-MS). Analytes were separated with a Phenomenx HYDRO-RP column (C18, polar endcapped; 50 mm × 2 mm, 4 μM), H_2O with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A and B, respectively. The flow rate was kept at 0.3 mL/min with the gradient as follows: 0 - 2 min 97% solvent A; 2 - 10 min 95% solvent B; 10 - 12 min 95% solvent B, 12 - 12.5 min 97% solvent A; 12.5 - 17 min solvent A. The MS was operated in a positive electrospray ionization mode. Concentration of OBZ was determined by an external standard calibration curve. Identification of OBZ metabolites was carried out by MS/MS fragmentation using nitrogen as the collision gas. The accurate mass of OBZ related compounds was further obtained with a LC system (ThermoFisher) coupled to an ultra-high resolution time of

flight mass spectrometer (Bruker). Mass transitions and characteristics of OBZ and metabolites are listed in Table 9.

Table 9 Retention time and proposed identities of OBZ and it metabolites (OBZ: oxybenzone; DHB: 2,4-dihydroxybenzophenone; OBZ-Mal-Glu: oxybenzone-(6-*O*-malonyl)-glucoside; OBZ-Glu: oxybenzone-glucoside)

Compound	Retention time (min)	Molecular formula	Precursor ion *m/z*	Fragment ions *m/z*
OBZ	10.8	$C_{14}H_{12}O_3$	229.0859 $[M + H]^+$	151.105 $[M + H]^+$
DHB	7.7	$C_{13}H_{10}O_3$	215.0715 $[M + H]^+$	137.105 $[M + H]^+$
OBZ-Mal-Glu	7.3	$C_{23}H_{24}O_{11}$	499.1223 $[M + Na]^+$	455.251 $[M + Na]^+$
OBZ-Glu	6.9	$C_{20}H_{22}O_8$	413.1218 $[M + Na]^+$	251.185 $[M + Na]^+$

2.3.4 *In vitro* synthesis/hydrolysis of OBZ metabolites

2.3.4.1 Enzymatic synthesis and hydrolysis of oxybenzone-glucoside

O-glucosyltransferase (GT) was extracted from plant material according to the protocol published previously (San Miguel et al., 2013). In short, 3 g of hairy roots were homogenized under liquid nitrogen with mortar and pestle and extracted with 100 mM sodium phosphate-buffer pH 6.5 containing 10 mM DTE, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 1% PVP K90 at 4°C for 30 min. After centrifugation at 15000 × g for 30 min at 4°C, proteins in the supernatant were precipitated by progressively adding ammonium sulphate to 40% and 75% saturation and centrifuged at 18500 × g for 30 min at 4°C respectively. Consequently, the pellets were re-suspended in 2.5 mL 200 mM Tris/HCl buffer with 2 mM $MgCl_2$ and 1 mM DTE, pH 7.3. Proteins were desalted by chromatography through PD 10 columns (GE Healthcare, UK) and stored at -80°C before use.

In vitro incubation with GT was performed by incubating 100 µM OBZ with 2 mM uridine diphosphate glucose, 1.25 mM 4-NPG, 1.25 mM salicin in 200 mM Tris/HCl buffer, pH 7.5. The reaction was started by adding 100 µL GT containing enzyme extract to yield a final volume of 200 µL, and incubation lasted for 1 h at 30°C (Messner et al., 2003). Experiments without enzyme served as control. The reaction was stopped by precipitating the protein with 10 µL concentrated phosphoric acid and centrifuged at 13000 × g for 2 min.

Enzymatic hydrolysis was started by incubating metabolite extracts (prepared after SPE, evaporated to dryness and re-constituted in 400 µL of 50 mM sodium phosphate buffer, pH 5.0) with 300 U of commercial β-D-glucosidase at 36 °C for 2 h. Control experiments were free of glucosidase. Reaction was stopped by applying samples to the SPE columns (Huber et al., 2012).

2.3.4.2 Alkaline hydrolysis of oxybenzone-(6-*O*-malonyl)-glucoside

Alkaline hydrolysis was performed by incubating plant extracts after SPE with 0.1 N NaOH for 6 h at room temperature, and samples without NaOH were controls (Petroutsos et al., 2007). All the samples were analyzed by LC-MS.

2.3.5 Protein extraction and antioxidative enzymes assay

The extraction of soluble protein was carried out according to the method described by Schröder et al. (2005), the procedure was similar to the GT protocol with some differences: the second precipitation reached 80% saturation and the following centrifugation was carried out at 20000 rpm; pellet was re-dissolved in 2.5 mL of 25 mM Tris/HCl buffer, pH 7.8.

All enzyme assays were conducted in a 96-well spectrophotometer (Spectra max Plus 384). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as the change from epinephrine to adrenochrome at 480 nm for 5 min. One unit of SOD activity is defined as the amount of protein required for 50% inhibition of adrenochrome formation (Polle et al., 1989). Catalase (CAT, EC 1.11.1.6) activity was measured by the decrease in absorption at 240 nm due to the consumption of H_2O_2 (Verma and Dubey, 2003). Peroxidase (POX, EC 1.11.1) activity was assayed at 420 nm using guajacol as a substrate (Diekmann et al., 2004.). Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was measured according to the method described previously (Lyubenova et al., 2015) with modifications. Each reaction contained 1mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and enzyme extract in 50 mM KH_2PO_4/K_2HPO_4 buffer, and the activity was determined at 290 nm.

2.3.6 Estimation of typical reactive oxygen species

O_2^- was detected through the formation of blue, water insoluble formazan. *Cyperus* leaves were incubated in the dark with 5 mL 0.25 mg/mL nitro blue tetrazolium chloride in 25 mM HEPES/KOH buffer (pH 7.6) for 2 h. H_2O_2 was detected by staining with 3,3'-

diaminobenzidine (DAB). Leaf samples were incubated in the dark with 0.25 mg/mL DAB in 50 mM Tris/Acetate buffer (pH 5.0) for 24 h. In both cases, samples were subsequently boiled in 95% ethanol for 30 min to remove chlorophylls and finally photographed by a stereomicroscope (Leica M125) equipped with an HD camera (Leica MC120). The images were analyzed by GIS-software (IDRISI Selva) to classify the intensity of staining.

2.3.7 Measurement of lipid peroxidation

Lipid peroxidation levels in *Cyperus* tissues were estimated indirectly in terms of malondialdehyde (MDA) content, by measuring thiobarbituric acid reactive species (TBARS) according to the methods of Heath and Packer (1968) with modifications. After extraction with trichloroacetic acid, the samples were incubated in a water bath at 95°C for 30 min, and then centrifuged at 10000 × g for 5 min. The fluorescent product was determined spectro-photo-metrically at 532 nm corrected for nonspecific turbidity by subtracting the absorbance at 600 nm (Spectra MAX 190 J, Molecular Devices).

2.3.8 Toxicity tests with plant seeds and *Lemna minor*

Toxicities of 5 µM OBZ and 3 mg/L TiO_2 were evaluated in seed germination tests by recording the germination rate and root length of seedlings of barley (*Hordeum vulgare*) and tomato (*Lycopersicon esculentum*). Seeds of both plant species were germinated on filter papers moisturized with distilled water and spiked with 5 µM OBZ or 3 mg/L TiO_2 or in combination, respectively. Tests with *Lemna minor* were conducted as described in 2.2.4. Root length of seedlings and frond area of *L. minor* were recorded by a commercial image processing software (ImageJ).

2.4 Statistical analysis

Statistically significant differences among samples were evaluated by ANOVA tests with Tukey pairwise comparisons or Bonferroni post-test (Graph-Pad Prism software v5.0). Comparisons were considered significantly different for $p < 0.05$. Data are given as means ± standard deviation; numbers of replicates are given in legends.

Chapter 3

3 Manuscript overview

This section summarizes four manuscripts and states the publication status and contribution of authors.

i. Manuscript 1

Chen F, Huber C, May R, Schröder P. Metabolism of Oxybenzone in a hairy root culture: perspectives for phytoremediation of a widely used sunscreen agent, Journal of Hazardous Materials (2016), 306: 230-236.

ii. Manuscript 2

Chen F, Schnick S, Schröder P. Concentration effects of the UV filter Oxybenzone in *Cyperus alternifolius*: assessment of tolerance by stress related response, Environmental Science and Pollution Research (under review).

iii. Manuscript 3

Chen F, Huber C, Schröder P. Fate of the sunscreen compound Oxybenzone in *Cyperus alternifolius* based hydroponic culture: uptake, biotransformation and phytotoxicity, Chemosphere (2017), 82:638-646.

iv. Manuscript 4

Chen F, Schröder P. Uptake and transformation of Oxybenzone in the presence of TiO_2: Impact of nanoparticles on the plant remediation of organic UV filter (in preparation).

Manuscript 1 Metabolism of Oxybenzone in a hairy root culture: Perspectives for phytoremediation of a widely used sunscreen agent

Feiran Chen, Christian Huber, Robert May, Peter Schröder

Published in Journal of Hazardous Materials (2016), pp. 230-236,

DOI 10.1016/j.jhazmat.2015.12.022

This manuscript investigates the metabolism of oxybenzone in plant tissues by using hairy roots cells of *Armoracia rusticana* (horseradish) as a model.

OBZ, known as Benzophenone-3, is a commonly used UV filter in sun tans and skin protectants, entering aquatic systems either directly during recreational activities or indirectly through wastewater treatment plants discharge. To study the potential degradation capacity of plants for OBZ in phytotreatment, a well-established hairy root culture (*Armoracia rusticana*) was treated with 100 μM OBZ. After 3 h incubation, roots were transferred to fresh growth medium free of OBZ. Samples were harvested at 2, 4, 6 and 24 h after transferring the cells. More than 20% of spiked OBZ was eliminated from the medium by hairy roots after 3 h of exposure. Two metabolites were identified as oxybenzone-glucoside (OBZ-Glu) and oxybenzone-(6-*O*-malonyl)-glucoside (OBZ-Mal-Glu) by LC–MS/MS and TOF–MS. Formation of these metabolites was confirmed by enzymatic synthesis, as well as enzymatic and alkaline hydrolysis. Incubation with *O*-glucosyltransferase extracted from roots formed OBZ-Glu; whereas β-d-Glucosidase hydrolyzed OBZ-Glu. However, alkaline hydrolysis led to cleavage of OBZ-Mal-Glu and yielded OBZ-Glu. In the hairy root culture, an excretion of OBZ-Glu into the growth medium was observed while the corresponding OBZ-Mal-Glu remained stored in root cells over the incubation time. We propose that metabolism of oxybenzone in plants involves initial conjugation with glucose to form OBZ-Glu followed by malonylation to yield OBZ-Mal-Glu, and the malonylation of glucoconjugate promoted efficient sequestration of metabolites in plants.

Remark:

Idea for the manuscript: F. Chen, C. Huber, P. Schröder
Treatment with OBZ: F. Chen; LC-MS/MS & Data analysis: F. Chen, C. Huber; R. May
Manuscript draft: F. Chen
All authors contributed to the comments on the manuscript.

Manuscript 2 Concentration effects of the UV filter Oxybenzone in *Cyperus alternifolius*: Assessment of tolerance by stress related response

Feiran Chen, Sandrine Schnick, Peter Schröder

Under review with Environmental Science and Pollution Research

This manuscript studies the influence of oxybenzone particularly oxidative stress on plants by applying a short term incubation of detached *Cyperus alternifolius* with OBZ.

Phytoremediation has been proposed to reduce the load of the sunscreen oxybenzone (OBZ) in aquatic environment. Despite the proven removal efficiency of this compound, little is known about its influence, particularly oxidative stress on plants. In this study, a short term incubation of macrophytic *Cyperus alternifolius* was performed to prove plant´s ability to withstand the stress. Detached shoots were immersed in medium spiked with different concentrations of OBZ (50, 100 and 500 µM) for 2, 4 and 7 days, respectively. OBZ gives rise to O_2^- formation and to the production of H_2O_2 in *Cyperus* characterized by intense colorization following histochemical staining. Alterations of enzyme activities involved in the antioxidative defense system indicate an adaptive response of *C. alternifolius* to this xenobiotic stress. Quantification of lipid peroxidation reveals that no significant membrane damage occurred during incubation with OBZ. Overall, 50 µM OBZ (ten-fold higher than the amount frequently detected in the environment) exhibited low toxic effects. Accordingly, this pilot study provides information on potential use of *Cyperus* to remove emerging sunscreen contaminants from water bodies.

Remark:

Idea for the manuscript: F. Chen, P. Schröder;
Plant treatment with OBZ: S. Schnick
Measurement and Data analysis: S. Schnick, F. Chen
Manuscript draft: F. Chen
All authors contributed to the comments on the manuscript.

Manuscript 3 Fate of the sunscreen compound Oxybenzone in *Cyperus alternifolius* based hydroponic culture: Uptake, biotransformation and phytotoxicity

Feiran Chen, Christian Huber, Peter Schröder

Published in Chemosphere (2017), pp. 638–646,

DOI org/10.1016/j.chemosphere.2017.05.072

This manuscript explores the accumulation and transformation of oxybenzone in the candidate macrophyte species *Cyperus alternifolius*, and shows the tolerance of this species to this xenobiotic compound.

Oxybenzone (OBZ), a common ingredient in sunscreens and personal care products, has been frequently detected in effluents from municipal wastewater treatment plants and also in surface waters. OBZ is an emerging contaminant due to its adverse impacts on marine/aquatic ecosystems. To investigate the removal and degradation capacity of phytotreatment for OBZ, the common wetland plant species *Cyperus alternifolius* L. was exposed to this compound at 5, 25 and 50 µM for 120 h, respectively. Continuous uptake by roots and accumulation in plant tissues was observed over the exposure time, and depletion of spiked OBZ from the aqueous medium exceeded 73.9 ± 9.1% after 120 h. Similar to its fate in mammalian cells, OBZ is activated in a phase I reaction resulting in the hydroxylated metabolite 2,4-dihydroxybenzophenone (DHB). Independently, two phase II metabolites were identified as oxybenzone-glucoside (OBZ-Glu) and oxybenzone-(6-O-malonyl)-glucoside (OBZ-Mal-Glu) by LC-MS/MS. Formation of these metabolites increased over the experimental period. To our knowledge this is the first time that DHB, OBZ-Glu and OBZ-Mal-Glu are shown to be formed in higher plant tissues. Furthermore, plant defense systems-antioxidative enzymes (SOD, CAT, APOX and POX) were found to be elevated to counteract stress caused by exposure to OBZ. This study presents the huge potential of aquatic plants to cope with benzophenone type UV filters in contaminated water bodies.

Remark:

Idea for the manuscript: F. Chen, C. Huber, P. Schröder;
Plant treatment with OBZ: F. Chen; LC-MS/MS & Data analysis: F. Chen, C. Huber
Manuscript draft: F. Chen
All authors contributed to the comments on the manuscript.

Manuscript 4 Uptake and transformation of Oxybenzone in the presence of TiO_2: Impact of nanoparticles on the plant remediation of organic UV filter

Feiran Chen and Peter Schröder

In preparation

This manuscript reveals the potential influence of TiO_2 on the plants encountered with OBZ at environmental related concentration.

Oxybenzone (OBZ) presents in the environment as an emerging contaminant may occur jointly with the nanoparticle TiO_2 due to the typical composition of sunscreens. Thus, the effects caused by TiO_2 must be considered when investigating the environmental fate of sunscreens and also when plant performance on remediation of OBZ is scrutinized. Toxicity effect of OBZ and TiO_2 on plant development was evaluated by germination rate and root length of tomato and barley. Results showed that OBZ significantly inhibited germination rate of tomato seeds, while no effect was observed for germination of barley seeds. Interestingly, co-exposure with TiO_2 lowered the toxicity of OBZ on the tomato seedlings as there were no differences on germination rate and root length between co-exposure and control treatments. Moreover, growth inhibition test with *Lemna minor* showed that addition of TiO_2 even enhanced growth rate by increasing the frond area. Furthermore, influence of TiO_2 (3 mg/L) on removal of OBZ (5 µM) by plants was examined with respect to the variations in uptake and metabolism of OBZ in a hairy root culture system. Co-exposure to TiO_2 amplified the accumulation of OBZ in plants; while transformation to OBZ metabolites was less efficient when TiO_2 had been added. Therefore, it can be concluded that Ti-nanoparticles may generally reduce the phyto-toxicity of OBZ and increase the uptake of this compound in phytoremediation, while the interaction on the transformation capacity should be considered when applying phytoremediation for UV-filter contaminated water.

Remark:

Idea for the manuscript: F. Chen, P. Schröder;
Plant treatment with OBZ: F. Chen; Measurement & Data analysis: F. Chen
Manuscript draft: F. Chen
All authors contributed to the comments on the manuscript.

Chapter 4

4 General discussion

The occurrence of pharmaceuticals and personal care products (PPCP) in water resources has become a concern and raised public attention due to their interference with the endocrine system of human and animal species. Since conventional wastewater treatment systems (WWTP) are not designed to treat these types of compounds, as a result the effluent from treatment plants becomes one of the main sources of emerging contaminants. Additionally, direct pollution from recreational activities is another important input source for emerging contaminants into water bodies. Given the high cost of installing and operating advanced treatments (UV photolysis, ion exchange, and membrane filtration), the use of plants for remediation may be more favorable, especially for small or outdated WWTP. The plant-based system can in these cases be applied as alternative for secondary treatment or as tertiary treatment to polish the effluent. In recent years, the elimination of PPCP by plant-based technologies has been increasingly explored (Zhang et al., 2014). However, detailed investigations about the fate and pathways of particular compounds in plants are scarce, since the literature is focused on removal efficiency rather than fate of the compounds. To shed some light on plant uptake, accumulation and transformation of xenobiotics, this thesis was conducted to address a possible green solution for reducing the potential negative impacts on the ecosystem.

This thesis shows the application of aquatic plants to eliminate the typical UV filter OBZ as an example, and contributes to the understanding of OBZ removal and behavior in plants. To demonstrate the potential of plant to uptake and transform OBZ, a short term treatment was performed by using a horseradish hairy root culture as a model (Manuscript 1). Manuscript 2 investigated the plant stress of the selected macrophyte species, *Cyperus*

alternifolius, with respect to OBZ, and concluded that only mild stress occurred in plant and 50 µM of OBZ generated the lowest negative impact. Based on these results, a hydroponic experiment was conducted to present the whole pathway of OBZ in entire plants of *C. alternifolius.* It exhibited the uptake of OBZ by roots and the distribution of this compound in different organs as well as the metabolism of OBZ in each tissue (Manuscript 3). To answer the question of OBZ removal efficiency under mixed pollution, the hairy root model system was exposed to OBZ and another inorganic UV filter, TiO_2 (Manuscript 4), where proof can be presented that co-exposure enhanced plant growth and elevated the uptake of OBZ by the plant.

4.1 Removal of OBZ by plants

Generally, the main removal mechanisms of xenobiotics postulated in hydroponic systems are abiotic (photodegradation, evaporation, hydrolysis) and biotic processes (plant uptake and microbial degradation) (Zhang et al., 2016). In agreement with previous findings that OBZ is resistant against abiotic processes such as irradiation (Rodil et al., 2009), OBZ concentration in the control vessels (no plant) remained constant over the experimental period (Manuscript 3). Direct uptake and accumulation of contaminants by plants are important mechanisms for phytoremediation (Collins et al., 2005). The experiment with sterile hairy roots treated with OBZ excluded the influence of rhizospheric microbes; hence the elimination of OBZ was attributed mainly to the presence of the plant alone.

The hairy root experiment indicated that plants might have a great potential to remove OBZ within short time. After 3 h exposure, 20% of OBZ (100 µM) was eliminated from the medium (Manuscript 1). In the hydroponic experiment, more than 73.9 ± 9.1% of the spiked OBZ was depleted within 120 h. These results are in line with previous studies in which a constructed wetland applied as secondary treatment system had high removal efficiencies (88 - 97%) for OBZ (Matamoros et al., 2007). In tertiary treatment systems composed of polishing ponds and surface flow constructed wetland (SFCW), a removal efficiency of 43 - 94% was reported for OBZ, among which the SFCW accounted for a removal of 53 - 77% (Matamoros and Salvadó, 2012). Therefore, it might be concluded that the plant accounted for the major elimination of OBZ.

The capacity of plant roots to take up pollutants is of primary importance for studying the fate of these compounds. The uptake of non-ionized compounds from hydroponic solution into plant roots has been demonstrated to be based on a passive diffusion process (Briggs

et al., 1982), which is largely governed by the hydrophobicity of a given chemical compound. The partition coefficient log K_{ow} has been adopted to evaluate the hydrophobicity, log K_{ow} is the ratio of the concentration of unionized compound between octanol and water (Burken and Schnoor, 1998; Pilon-Smits, 2005). Lipophilic compounds show greater tendency to partition into roots than hydrophilic compounds, and mildly hydrophobic compounds are able to move through the lipid bilayer membrane and get into the plant (Collins et al., 2005; Pilon-Smits, 2005). OBZ is a neutral compound (Matamoros et al., 2007) with moderate hydrophobicity (Log K_{ow} 3.52) which may have great potential to be taken up into the plants.

4.2 Accumulation of OBZ in plants

The distribution and behavior of OBZ in *C. alternifolius* was studied in a hydroponic experiment (Manuscript 3). After exposure to three concentrations of OBZ independently, OBZ was taken up continuously by the roots and finally reached a concentration of 27.8 - 162.4 µg/g fresh weight (FW), which accounted for 19.9 - 58.4% of the total OBZ detected in *Cyperus*. This is supported by the prediction for efficient uptake of neutral compounds which have a log K_{ow} between 0.5 and 3.5 (Briggs et al., 1982; Schröder et al., 2008). Translocation of organic neutral compounds from root to shoot means that the molecule needs to pass the endodermis between root apoplast and the symplast with the xylem, with its casparian strip. This entry into the xylem is either mediated by transporters or driven by passive movement and is similar to the diffusion through a membrane. Thus again, compounds with optimal hydrophobicity are transported more efficiently to the xylem for further translocation with the transpiration stream (Briggs et al., 1982; Dietz and Schnoor, 2001; Pilon-Smits, 2005; Trapp, 2004). In agreement with this theory, OBZ was observed in the upper parts of *Cyperus*, but concentrations in shoots were lower than that in rhizomes and roots. In stems OBZ was detected at concentrations of 1.9 - 34.2 µg/g FW, and next to the rhizomes, stems accounted for the second largest amount of OBZ accumulated in *Cyperus* which could be attributed to its high biomass. Compared to the underground parts and stems, OBZ concentration in leaves was much lower (0.61 - 4.05 µg/g FW). The translocation in the xylem is driven by the transpiration which creates negative pressure to pull up the water and its solutes, therefore, the potential of translocation to the shoots is determined by hydrophilicity of the compound (Pilon-Smits, 2005). Briggs and co-workers (1982) have pointed out the maximum translocation

occurred at the optimum lipophilicity centered at Log K_{ow}=1.8. On this basis, OBZ might be too hydrophobic for efficient translocation to the top part of *Cyperus*.

4.3 Plant detoxification pathways for OBZ

The accumulation of xenobiotics in tissues may often cause problems of cytotoxicity to the plants. To prevent and reduce the harm resulting from the contact with xenobiotic compounds, in plants detoxification pathways have evolved which comprise of the "Green Liver concept" and the activation of antioxidant enzymes (Sandermann, 1992; Schwitzguébel et al., 2011).

In analogy to the mammalian liver, plants are hence considered as "green liver" to metabolize organic compounds, among them also anthropogenic pollutants, to frequently less toxic end products (Sandermann, 1992; Schröder, 2006). As presented in Fig. 8, after uptake into plants, the xenobiotic compounds may undergo modifications (oxidation, reduction, hydroxylation, etc.) to be more water soluble and available for the following conjugation (phase I). Subsequent reactions with endogenous compounds such as conjugation to glutathione catalyzed by glutathione S-transferases is of great importance to detoxify foreign compounds (Schröder et al., 2008), alternatively, conjugation to activated glucose or to a malonyl group also plays a role in the tolerance towards xenobiotics (phase II). Consequently, the conjugates are transported to the vacuole or bound to cell walls (Pilon-Smits, 2005; Sandermann, 1992; Zhang et al., 2014). So far, OBZ has been shown to be metabolized by phase I and II reactions in animals (Okereke et al., 1994). The location and toxicity of the final transformation products are of importance when considering the harvesting of plants to avoid re-entry of contaminants into the environment via dead plant material. Thus it is crucial to investigate the metabolism of OBZ in plants to improve phytoremediation procedures.

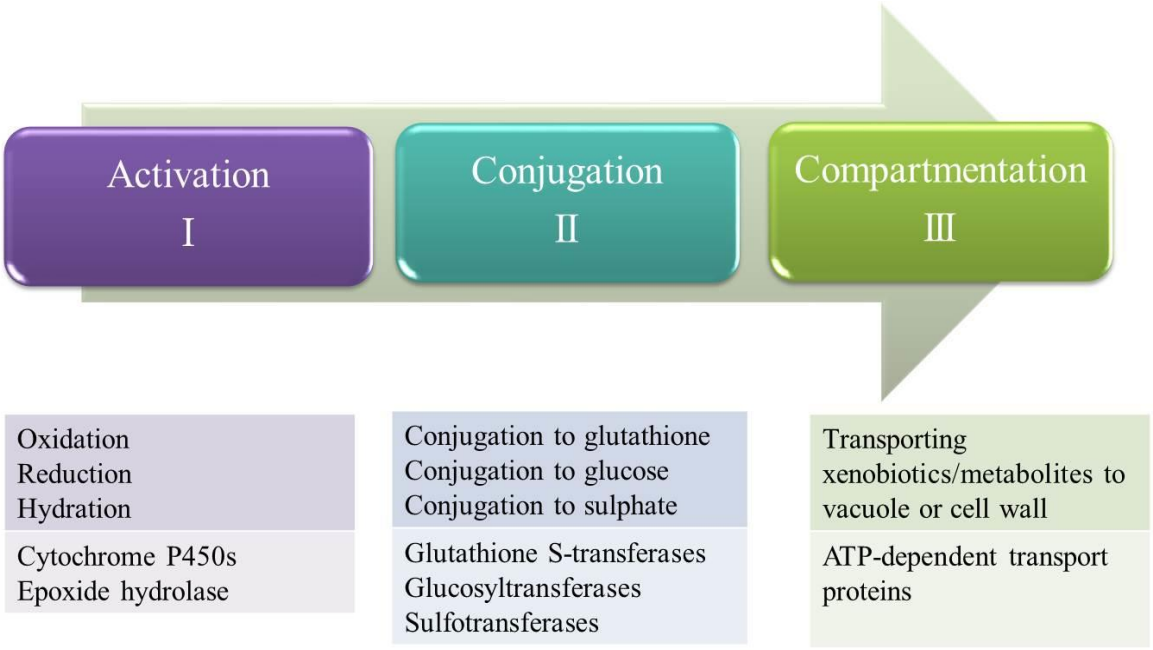


Figure 8 Transformation of organic xenobiotics in plants

Exposure to xenobiotics has in most cases been followed by the formation of reactive oxygen species (ROS) in plants such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Mittler, 2002). The over-accumulation of ROS results in the oxidative damage to plant cells such as membrane lipid peroxidation, protein oxidation and DNA mutation (Mittler, 2002). To keep the balance between production and elimination of ROS in plants, antioxidant enzymes are activated or even overexpressed. Superoxide dismutase (SOD) is at the first line of defense to convert O_2^- to H_2O_2. H_2O_2 can easily enter cell cytoplasm and form OH^- which attacks DNA and initiates lipid peroxidation. Catalase (CAT), ascorbate peroxidase (APOX) and peroxidase (POX) are key enzymes for the conversion of H_2O_2 to water (Schwitzguébel et al., 2011). Another important enzyme system is the ascorbate-glutathione cycle which detoxifies H_2O_2 and regenerates glutathione (Asada, 2006).

4.3.1 Transformation of OBZ

The hairy roots experiment exhibited the potential to transform OBZ after 3 h incubation. The sequence of transformation of OBZ was clearly observed in the subsequent incubation free of OBZ. After 3 h treatment with OBZ, two metabolites were detected; they were identified as oxybenzone-glucoside (OBZ-Glu) and oxybenzone-malonyl-glucoside (OBZ-Mal-Glu) for the first time in plants, to the best of our knowledge. OBZ-Glu is formed via

a direct conjugation to glucose catalyzed by an *O*-glucosyltransferase (E.C. 2.4.1.x); this gluco-pyranoside is further transformed via esterification with malonic acid catalyzed by a malonyltransferase (E.C. 2.3.1.x) (Manuscript 1). Metabolism of OBZ in mammalian cells has been reported mainly as demethylation, hydroxylation and conjugation with glucuronide and sulfate (Fediuk et al., 2012; Okereke et al., 1994; Wang et al., 2013). In hairy roots, the direct glucosylation without any previous chemical modifications could be attributed to the already existing hydroxyl group of OBZ. After 3 h pre-incubation with OBZ the roots were transferred to the OBZ free media. With the given amount of OBZ in roots, a difference between the amounts of both metabolites was observed. The formation of OBZ-Glu slowed down and reached after 24 h a level only 2.6-fold higher than in the pre-incubation phase, whereas the amount of OBZ-Mal-Glu increased 16.3-fold in the same time and became dominant. Conjugates with glucose and malonic acid have been shown to be less toxic and more water soluble than the respective parent compound (Bártíková et al., 2015). But studies have shown that not all glucosides are stable since they can be easily converted back to the parent compound by cytoplasmic glucosidase under certain conditions (Brazier-Hicks and Edwards, 2005; Morant et al., 2003), whereas malonylation of glucosides enhances their stability and promotes further retention of metabolites into the vacuole (Taguchi et al., 2010; Zhao et al., 2011). This implies that plants have high potential to metabolize OBZ and stabilize the metabolites in short time.

In the higher plant *Cyperus*, 2,4-dihydroxybenzophenone (DHB) was detected as a novel metabolite in addition to OBZ-Glu and OBZ-Mal-Glu. DHB is an activated metabolite of OBZ which forms via demethylation of the methoxy substituent (*O*-demethylation) (Okereke et al., 1994; Watanabe et al., 2015). Demethylation has also been identified as the major route of OBZ phase I metabolism in human (Wang and Kannan, 2013). This reaction is known to be mediated by the cytochrome P450 enzyme system which is capable to metabolize a variety of xenobiotics. Watanabe et al. (2015) suggested the formation of DHB was catalyzed mainly by the cytochrome P450 isoforms 1A1, 2C19, 1A2 in human and 2C6, 1A1 1A2 in rat, respectively. No specific plant P450 has been identified so far. In *Cyperus* exposed to 5 - 50 µM OBZ, DHB only accounted for 1 - 14% of the total mass of metabolites, indicating that conjugation rather than demethylation is the main detoxification reaction for metabolism of OBZ in plants. This result is supported by findings in mammals where conjugation appears to be effective for OBZ metabolism as most of the urinary OBZ occur in the conjugated form, while the proportion of phase I

derivatives only accounted for 25 - 37%. Among them DHB was found to be the major intermediate which indicates the easy conversion of OBZ to this metabolite (Wang et al., 2013). Conjugation with glucuronic acid catalyzed by glucuronosyltransferase has been reported to be the major phase II elimination pathway for OBZ in rats (Okereke et al., 1994). Plant glucosyltransferase and malonyltransferase have no mammalian counterpart, however the malonyl-glucosyl double conjugate may likewise correspond to the analogous mammalian glucuronosyl-conjugate (Sandermann, 1992), which shows a similarity between animal and plant in the metabolism of OBZ. In animals xenobiotic conjugates are eliminated from the body through excretory systems. Whilst plants do not have such excretion pathways, other mechanisms are required. Literature demonstrates that xenobiotic conjugates usually undergo further breakdown steps including incorporation of metabolites into the cell wall or the apoplast, or they are sequestered in the vacuole. In this context, OBZ-Mal-Glu is proposed to be compartmented into the vacuole since the malonyl residue has been observed to serve as a signal for transport into the vacuole (Schmidt et al., 1988; Schröder, 2006; Wink, 1997). Therefore, it is crucial to understand which type of primary conjugation occurred as this determines the final of fate of the compound (Schröder, 2006).

It is known that differences of metabolic activation vary according to plant species. In this context, horseradish hairy root culture and *Cyperus* exhibited different potentials for transforming OBZ. The variation between the transformation products indicates that the metabolism of OBZ is controlled by different enzymatic systems, which may have variable expression levels and substrate specificity in different plant species (Agostini et al., 2013). Based on the identified products, the transformation pathways of OBZ in plants have been proposed as shown in Figure 9.

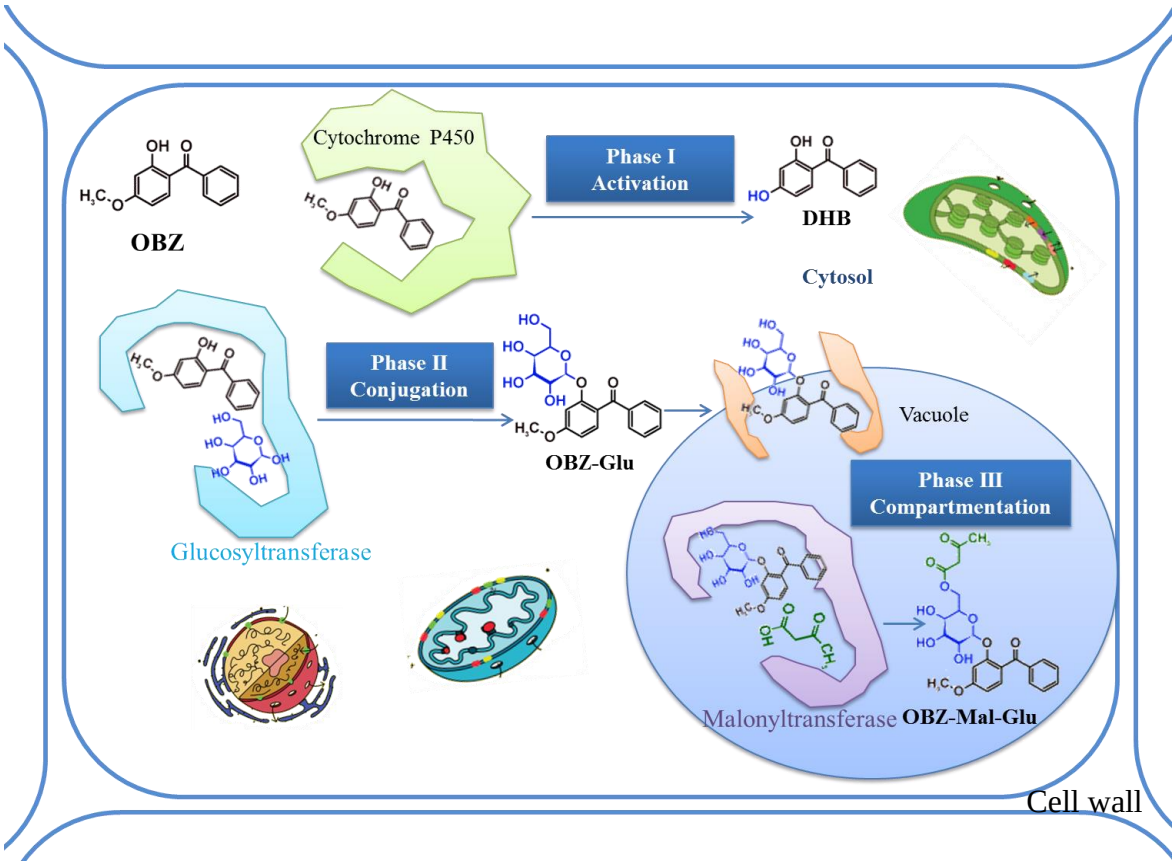


Figure 9 Proposed enzymatic pathway for the transformation of OBZ in plants (adapted from Sandermann, 1994; Van Aken, 2008; Dunn, 2012; Plengdut, 2017)

4.3.2 Phytotoxicity of OBZ

Manuscript 2 visualizes the formation of ROS (O_2^- and H_2O_2) in *Cyperus* exposed to OBZ. Higher amounts of ROS were observed for higher OBZ concentrations and longer incubation time. In plants, the induction of antioxidative enzyme activities is a general adaptation strategy to overcome oxidative stress. In accordance with the increasing formation of ROS, activities of enzymes responsible for scavenging the ROS content were induced to enhance stress tolerance, such as CAT, glutathione reductase (GR) and SOD, the activities of which were found to be elevated to 150 - 500% of control after two-day incubation. However, some enzyme activities were suppressed at higher concentrations, which is probably due to cell damage caused by the redox activity of ROS, indicating the limitation of *Cyperus* to tolerate higher doses of OBZ. On the other hand, the results also showed that OBZ concentration as much as 50 µM did not cause damage to the antioxidative system in *Cyperus*. Based on these findings, the experiment with the entire

plant proceeded further with lower OBZ concentrations as that found in the environment. During the exposure period, only mild stress was observed and no apparent symptom could be detected. Interestingly correlation was found between the activities of defense enzymes and OBZ concentrations accumulated in tissues, supporting the assumption that the enhancement of enzyme activities was caused by permanent uptake of OBZ.

Many phytotoxicitiy studies have focused on single chemical exposure (Yakubu, 2017), to evaluate responses under more realistic conditions, another preliminary screening test was carried out to assess the potential risks of multiple exposure with OBZ and TiO_2 during plant development (Manuscript 4). OBZ showed remarkable inhibition to tomato seed germination, and this lethal effect disappeared during co-exposure with TiO_2. A similar trend was found for the root elongation in the corresponding tomato seedlings. This beneficial effect of TiO_2 has also been shown in *Arabidopsis thaliana* exposed to the antibiotic tetracycline, when the phyto-toxicity of tetracycline on root elongation and biomass was alleviated by the presence of TiO_2. A possible explanation is that Ti-nanoparticles interact with tetracycline outside of the plant, thus preventing the exposure at a micro-/nano-level; also, co-exposure increased plant total protein content which plays an essential role in plant growth such as root elongation (Liu et al., 2017). Studies with tomato grown in multi-walled carbon nanotubes amended medium showed enhanced germination rates and growth as well, and it was presumed that the nanomaterial has affected the expression of genes that facilitate cell division and development (Khodakovskaya et al., 2013). Another proposed mechanism is the facilitated water uptake due to the increasing number of surface defects on the seed coat by the nanoparticles (Vithanage et al., 2017). The favorable effect was more obvious when incubating single fronds of *Lemna minor* in a 96-well plate based assay, where addition of 3 mg/L of TiO_2 increased frond area and photosynthetic pigments. Similar stimulation of *Lemna* growth has been reported in treatments with TiO_2 below 200 mg/L (Song et al., 2012). It has also been evidenced that TiO_2 could improve the resistance to aging of chloroplasts of spinach and could lower the production rate of free radicals. It has been further postulated that TiO_2 might directly clear a large amount of O_2^- by the reduction to H_2O_2 which can finally be cleaved by the antioxidative enzymes (Hong et al., 2005).

4.4 Impact of mixed exposure on remediation of OBZ by plant

Exposure to multiple pollutants is generally concomitant in the environment. Nanoparticles have been reported to alter the bioavailability of other coexisting contaminants and favor the entry of biomolecules into the plant cell. Based on the fact of possible co-occurrence of OBZ with TiO_2, manuscript 4 investigated the uptake of OBZ with respect to TiO_2. Hairy roots under co-exposure to OBZ and TiO_2 showed significantly higher amount of OBZ inside the exposed tissue. Previous results have shown that nanoparticles may induce the transport of secondary pollutants (Larue et al., 2012; Wild and Jones, 2009). For example, uptake of arsenic (As) has been enhanced by the presence of graphene oxide (GO) nanomaterial via at least three pathways, including the enhanced permeability due the structural damage to the cell wall, an up-regulation of transporter for As, and the co-transport of As that had been absorbed on GO (Hu et al., 2015). Also, Ma and Wang (2010) observed an increased (26 - 82%) accumulation of trichloroethylene in poplar upon exposure to fullerene nanomaterial under hydroponic conditions. Moreover, studies have provided evidence of facilitated bioaccumulation of nanoparticles in aquatic organisms. The vector-function of C_{60} nanoparticles was demonstrated in daphnids exposed to phenanthrene (Baun et al., 2008). Similar results were found in carp exposed to Cd-contaminated water, where the presence of TiO_2 nanoparticles led to an increase of Cd accumulation by 146%. This facilitated transport of Cd was attributed to the strong adsorption of Cd onto TiO_2, and Cd was accumulated into carp along with the accumulation of TiO_2. In accordance with this finding a positive correlation between Cd concentration and TiO_2 concentration has been confirmed with a correlation coefficient greater than 0.975 (Zhang et al., 2007).

OBZ-Glu and OBZ-Mal-Glu were again found to be the major metabolites in OBZ exposed roots. Correspondingly, activities of enzyme (GT) responsible for the metabolism of OBZ were elevated in OBZ and OBZ & TiO_2 co-exposure treatments. While transformation of OBZ was less efficient after addition of TiO_2, which was demonstrated by the lower GT compared to that in the OBZ alone treatment. Similar results were reported in *Arabidopsis* exposed to co-existing TiO_2 and trichloroethylene, where activities of antioxidative or stress-related enzymes (monodehydroascorbate reductase, peroxidase, γ-glutamylcysteine synthetase, glutathione S-transferase) were lower than that in the single treatment with either TiO_2 or trichloroethylene (Liu et al., 2017). Similarly, the generation of dimethylarsinate produced from detoxification of inorganic As has been inhibited by

graphene oxide (GO) nanomaterial, which led to the accumulation of highly toxic As (III) in wheat roots. The transformation of As(V) to As (III) was amplified in co-exposure to 0.1 or 1 mg/L GO, as the expression of arsenate reductase which catalyzes this transformation was increased, however the activity of this enzyme was inhibited at GO concentration of 10 mg/L, indicating a reduced toxicity (Hu et al., 2015). Also, in this study, GO was shown to inhibit the carbohydrate metabolism. This disturbance could be adopted to explain the alteration of conjugation of OBZ with glucose, as the substrate UDP-glucose for the glucosylation is involved in the carbohydrate metabolism.

Chapter 5

5 Conclusion

This study clearly showed the high importance of uptake and transformation of the target compound oxybenzone by hydroponic plant-based systems. UV filters are emerging contaminants to our environment due to the dual inputs from incomplete removal by the WWTPs and the direct contamination during recreational activities. This specific input situation has raised specific concern because the sources are not easy to control, and the spread of the compounds is ubiquitous. Oxybenzone as a typical UV filter has been shown to pose high risk potential to the aquatic organisms, and phytoremediation is considered as a promising alternative treatment for this compound.

Horseradish hairy root culture is an effective model to understand/predict the metabolic pathway of OBZ without interference of microbes or other plant parts. The transfer of OBZ-treated hairy roots to the clean medium showed the sequence of transformation of metabolites in plant. OBZ was partially metabolized to less toxic OBZ-Glu followed by secondary conjugation which formed OBZ-Mal-Glu. Malonylation of the glucoconjugate stabilized the transformation of OBZ and enhanced the compartmentation of metabolites in plants.

The study with the aquatic hydroponic system of *Cyperus alternifolius* contributed to the understanding of direct uptake, accumulation and translocation of OBZ by higher plants. OBZ was hydrophobic enough to move through the lipid bilayer of membranes, and accumulation of OBZ in underground parts was higher than that in stems. Translocation of OBZ to the top part of the plant was less efficient and consequently, lower concentration was observed in leaves. The occurrence of the same conjugates in horseradish and the aquatic *Cyperus* reiterated the importance of conjugation as a fate process in OBZ metabolism.

Activation of stress defense systems indicated the ability of the investigated macrophyte to counter the phytotoxicity caused by lower OBZ concentration. Additionally, the decreased toxicity in OBZ exposed plants upon addition of nanoparticle TiO_2 is an important step to better estimate the potential exposure risks of OBZ in real environment. Furthermore, simultaneous treatment with TiO_2 assisted the uptake of OBZ into plant. These findings provide useful information for enhancing the performance of such plant based pollution treatment.

Considering the particular emphasis on the occurrence of OBZ in the environment, the data obtained in this thesis serve as an important step forward for understanding the basic elimination and transformation processes that drive the removal of OBZ, and provide recommendations for purifying water associated with organic and inorganic UV filter contaminants.

Chapter 6

6 Outlook

The results in this thesis marked the beginning of understanding the fate of oxybenzone in plants. Large-scale and long term field work with multicomponent biological interactions is required for the reliable and effective implementation of the wastewater treatment technologies with phytoremediation. Considering the concentration dependent impact of nanomaterials on the uptake and transformation of secondary pollutants (Hu et al., 2015), further studies on the co-exposure to different concentrations of TiO_2 would be helpful for investigating the optimal removal and metabolism of OBZ. On the other hand, it is necessary to understand the antagonistic or synergistic effects of multiple pollutants on their accumulation and detoxification under both laboratory and real field conditions

Additionally, investigations addressing the final fate of metabolites and their impact on the environment remain to be explored. It is particularly important to note that plants rarely mineralize organic compounds, and conjugation does not result in complete detoxification of xenobiotics, while microorganisms have the potential to breakdown the compounds. Plant associated endophytic bacteria have been shown to promote the growth of host and contribute to the strong degradation of xenobiotic compound (Sauvêtre et al., 2018; Sauvêtre and Schröder, 2015). Biodegradation by microorganisms can be independent of their effect on plant growth, and microorganisms may act synergistically with each other for contaminant degradation (Thijs et al., 2017). In this context, it can be speculated that endophytes in living as well as decaying plant material or in plant litter might still be active and play important roles. Therefore, studies on the interactions between plant and the associated rhizospheric and endogenous microbiota are of special interest, and particular attention should be paid on the role of microorganisms during the post-remediation stage. Such comprehensive ecological and biochemical studies will further improve the performance of phytoremediation.

To date, root exudate compounds are known as inducers or inhibitors of metabolism pathways, but they are also substrates for microbial growth. This highlights the need to pinpoint a group of root exudates which directly stimulate the degradation of contaminants and favor the corresponding mineralization by microorganisms. This may eventually lead to a rationale design of exudate solutions which can be applied as a kind of fertilizers to facilitate the remediation.

Further, when adding phytotreatment to existing WWTP systems as either a supplement or eventual replacement, the effects of both systems need to be considered in combination with each other and over time as the phyto-technology system matures at the site (ITRC, 2009). The market for phyto-technologies is expected to grow during the coming years. To gain greater acceptance from industry and government, the economic opportunities in the post-harvest management of phytoremediation should be developed, and the increased value coming from making best use of the harvested plant material will in turn make the technology more commercially attractive and improve the ecological interest of phytoremediation of contaminated fields. In any case, plant metabolism provides a valuable toolbox for the removal of unwanted chemicals from our environment and the limited water resources.

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Appendix A

Manuscript 1 - 4

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Metabolism of oxybenzone in a hairy root culture: Perspectives for phytoremediation of a widely used sunscreen agent

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HIGHLIGHTS

- First finding on degradation of oxybenzone in plant tissues.
- Two novel metabolites were identified.
- Glucosylation and malonylation are main Phase II mechanisms.
- Phytoremediation exhibits good potential for treating sunscreen compounds.

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ABSTRACT

Oxybenzone (OBZ), known as Benzophenone-3, is a commonly used UV filter in sun tans and skin protectants, entering aquatic systems either directly during recreational activities or indirectly through wastewater treatment plants discharge. To study the potential degradation capacity of plants for OBZ in phytotreatment, a well-established hairy root culture (*Armoracia rusticana*) was treated with OBZ. More than 20% of spiked OBZ (100 μM) was eliminated from the medium by hairy roots after 3 h of exposure. Two metabolites were identified as oxybenzone-glucoside (OBZ-Glu) and oxybenzone-(6-*O*-malonyl)-glucoside (OBZ-Mal-Glu) by LC–MS/MS and TOF–MS. Formation of these metabolites was confirmed by enzymatic synthesis, as well as enzymatic and alkaline hydrolysis. Incubation with *O*-glucosyltransferase (*O*-GT) extracted from roots formed OBZ-Glu; whereas β-D-Glucosidase hydrolyzed OBZ-Glu. However, alkaline hydrolysis led to cleavage of OBZ-Mal-Glu and yielded OBZ-Glu. In the hairy root culture, an excretion of OBZ-Glu into the growth medium was observed while the corresponding OBZ-Mal-Glu remained stored in root cells over the incubation time. We propose that metabolism of oxybenzone in plants involves initial conjugation with glucose to form OBZ-Glu followed by malonylation to yield OBZ-Mal-Glu. To our best knowledge this first finding presenting the potential of plants to degrade benzophenone type UV filters by phytoremediation.

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1. Introduction

Personal care products including stimulants, fragrances, sunscreens, antimicrobials, and insect repellents are emerging contaminants which have attracted much attention in recent years due to their presence in surface water and potential effects on ecosystems [1,2]. Only recently, UV sunscreens have become a topic of environmental research because they appear in cosmetic products up to 6% and may also be used as indirect additives in food contact substances [3,4]. Contamination of the aquatic environment by UV filters occurs either indirectly from wastewater treatment plant discharges, or directly via recreational activities due to the release of chemical compounds from skin to water.

OBZ is one of the most frequently found UV filters detected in wastewater, swimming pool water but also in surface water samples, occurring at concentrations ranging from 68 to 3300 ng L^{-1} [5–9]. These findings indicate that OBZ is not fully eliminated during wastewater treatment and may be disseminated further into the environment. Hence, OBZ has been marked as an emerging contaminant since 2005 in Richardson's water analysis [10]. Studies demonstrate that OBZ and its metabolites accumulate in aquatic organisms (perch, roach and juvenile rainbow trout)

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[11,12], and may induce endocrine disrupting activities including antiestrogenic and antiandrogenic activities [13–15]. A recent paper even indicated that bleaching of coral reefs might be related to the release of sunscreens containing OBZ in coral reef areas [16].

Amongst attempts to reduce the amount of unwanted compounds in the environment, phytoremediation as an emerging technology which involves uptake, degradation and storage of contaminants by plants has been proposed. Roots are the main organs for absorption and transformation reactions [17]. In this context, hairy root cultures have been applied as a reliable experimental model to illustrate the metabolic processes and fate of contaminants in whole plants during phytoremediation. Products identified in hairy roots are considered to be formed in the roots of intact plants as well [18]. Hairy roots cell cultures have advantages of fast growth, free of photo- and microbial degradation, easy implementation and high production of secondary metabolites [19].

Plant cells cope with foreign compounds mainly in successive phases which have been described by Sandermann's "Green Liver" concept [20]. Phase I activates compounds by oxidation, reduction or hydroxylation for the subsequent conjugation to reactive groups such as amino acids, sugars and peptides by phase II enzymes. Phase III (sequestration) mostly refers to further conjugation or metabolism of phase II products for storage in vacuoles or cell walls [20,21].

In phase II, glucosylation is one of the most commonly observed detoxification mechanisms, which is employed by many organisms to maintain metabolic homeostasis. It is catalyzed by glucosyltransferases (E.C. 2.4.1.x) attaching activated glucose on a xenobiotic acceptor molecule. By addition of sugars, the interactive aglyca are converted into stable, more water soluble and less toxic forms [22]. Plants are known to glucosylate a diverse range of endogenous and exogenous organic molecules, such as flavonoids, herbicides, pesticides and other xenobiotics [23–27]. *O*-glucosides are often further metabolized to form a malonate hemi-ester conjugate with the involvement of malonyl-CoA transferase (E.C. 2.3.1.x) to add an *O*-malonyl substituent to a hydroxyl group of a sugar residue of xenobiotics [20]. Malonylation is characterized by enhanced chemical stability, improved solubility and deposition of target compounds in vacuoles [28]. Malonylation of glucosides has been reported in several plant species including thale cress, tobacco, common duckweed, butterfly pea, and soybean [23,29–31].

So far, studies on the transformation of OBZ are mainly limited in mammals, hence, in this study we investigate the metabolism of OBZ in plant tissues by using hairy roots cells of *Armoracia rusticana* (horseradish) as a model. Structures of key metabolites are proposed and the formation of metabolites is presented as a function of time, and a recommendation on the phytoremediation potential is given.

2. Materials and methods

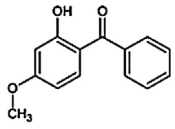
2.1. Chemicals

Oxybenzone (2-Hydroxy-4-methoxybenzophenone, pharmaceutical secondary standard) was purchased from Fluka (Germany), acetonitrile (HPLC grade), water with 0.1% formic acid (LC–MS grade), acetonitrile with 0.1% formic acid (LC–MS grade) were obtained from Carl Roth (Germany). All water used for sample preparation was ultrapure (MilliQ, Millipore Corporation). Physiochemical properties of OBZ are shown in Table 1.

2.2. Plant material

Horseradish (*A. rusticana* L.) hairy root culture transformed by *Agrobacterium rhizogenes* strain A4 [32] was grown in 100 mL full-strength Murashige and Skoog medium containing thiamine and inositol for 10 days. OBZ was dissolved in ethanol and added to the growth medium to yield a final concentration of 100 μM. After 3 h of treatment with OBZ, roots were washed twice with sterilized deionized water and transferred to fresh growth medium without OBZ. Samples were harvested at 2, 4, 6 and 24 after transferring the cells, dried with lint tissue paper, frozen in liquid nitrogen and stored at −20 °C.

Table 1
Chemical structure and physicochemical properties of oxybenzone.

Chemical structure	Molecular formula	Molecular weight	pK_a
	$C_{14}H_{12}O_3$[a]	228.24328 g/mol[a]	8.07 and −4.8[b]

[a] Pubchem.
[b] DrugBank.

2.3. Oxybenzone and metabolites extraction and sample preparation

0.5 g root material were ground under liquid nitrogen, and extracted with 1.5 mL H_2O/Acetonitrile (30/70, *v*/*v*). Samples were vortexed for 1 min, treated in the ultrasonicator for 5 min and centrifuged at 13,000 × *g* at 4 °C for 30 min. Supernatants were collected and filtrated through 0.45 μm pore size PVDF syringe filters (Carl Roth, Germany) prior to loading on them on solid phase extraction (SPE) columns (3 cm^3 60 mg Oasis HLB SPE cartridges, Waters, Germany) for further purification. Cartridges had been conditioned with 3 mL of methanol and equilibrated with 3 mL of water prior to use. Samples (0.5 mL) were passed through the cartridges and 3 mL water were used to flush impurities. The cartridges were then dried under vacuum for 10 min, and analytes were subsequently eluted with two 0.7 mL and one 0.6 mL aliquots of acetonitrile. For the detection of OBZ and metabolites in medium, 200 μL of growth medium was filtered through PVDF syringe filters as mentioned above.

2.4. LC–MS analysis

LC–MS analysis was performed with a HPLC system (Varian ProStar 210) coupled to an ion trap mass spectrometer (Varian 500-MS) as described elsewhere [33]. A Phenomenex HYDRO-RP column (C18, polar endcapped; particle size 4 μm; 50 mm × 2.0 mm) was applied for rapid separation of analytes using the following mobile phases for elution: H_2O, 0.1% formic acid as mobile phase A and acetonitrile, 0.1% formic acid as mobile phase B with following gradient: 0–2 min 97% Buffer A (isocratic); 2–10 min 95% Buffer B (linearly increasing); 10–12 min 95% Buffer B (isocratic); 12–12.5 min 97% Buffer A (linearly decreasing); 12.5–17 min 97% A (isocratic). The flow rate was 0.3 mL/min. Concentration of OBZ was determined by an external standard calibration curve. The HPLC eluent was introduced to the mass spectrometer using a pneumatically assisted electrospray source. The mass spectrometer was operated in positive ESI mode. The interface was adjusted to the following conditions: capillary voltage, 63 V; needle voltage, 4500 V; drying gas temperature, 350 °C. MS/MS spectra were obtained by collision-induced dissociation using nitrogen as the collision gas. Mass transitions of OBZ and metabolites were as follows: $[M+H]^+$ *m/z* 229–151 (oxybenzone), $[M+Na]^+$ *m/z* 413–251 (oxybenzone-glucoside), $[M+Na]^+$ *m/z* 499–455 (oxybenzone-malonyl-glucoside).

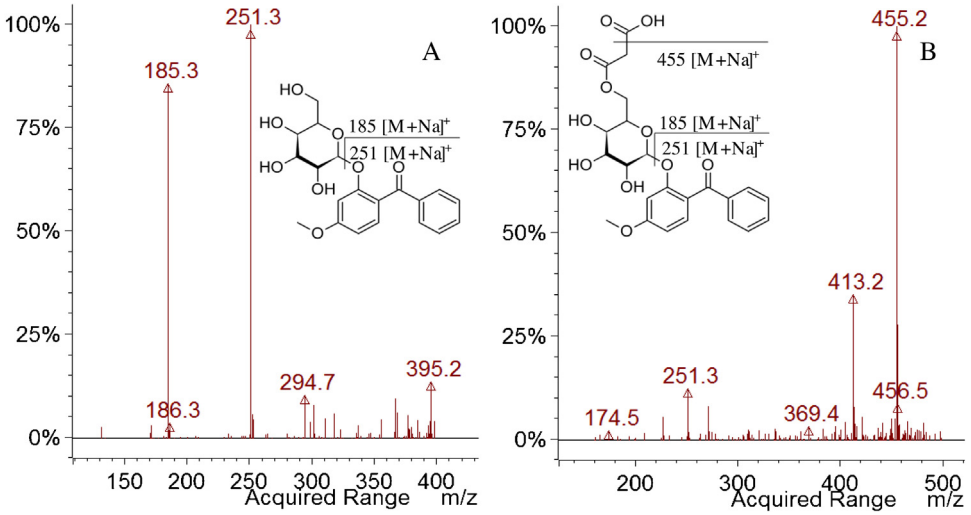


Fig. 1. MS/MS spectra of $[M+Na]^+$ of A OBZ-Glu (*m/z* 413) and B OBZ-Mal-Glu (*m/z* 499) from hairy root extracts. The product ion *m/z* 251 is formed via a cleavage of dehydroglucose; a loss of CO_2 resulted in the formation of *m/z* 455, and the cleavage of the ester bond released free OBZ-Glu (*m/z* 413). The analysis was done in positive ionization mode. (2-column fitting).

LC–TOF–MS experiments were conducted on an Ultimate 3000 LC system (ThermoFisher) coupled to an ultra high resolution Maxis 4 g plus TOF mass spectrometer (Bruker) equipped with an electrospray source. The LC conditions were identical as above. The TOF–MS was operated in positive polarity mode with active focus under the following conditions: Capillary voltage, 5500 V; nitrogen dry gas temperature, 225 °C; dry gas flow, 10 L/min; nebulizer pressure, 2 bar. The TOF–MS was calibrated daily with ESI-L tuning mix (Agilent) using the enhanced quadratic algorithm. MS scans were recalibrated using Hexakis (1H, 1H, 4H-hexafluorobutyloxy) phosphazine (Agilent) as a lock mass.

2.5. Enzyme extraction

Extraction of an *O*-glucosyltransferase containing enzyme fraction was conducted with some modification as previously described [26]. In short, 3 g of hairy roots were homogenized in liquid nitrogen with mortar and pestle to yield a fine powder and extracted with 30 mL 100 mM sodium phosphate-buffer pH 6.5 containing 10 mM DTE, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 1% PVP K90 at 4 °C for 30 min. After centrifugation at 15,000 × *g* for 30 min at 4 °C, proteins in the supernatant were precipitated progressively by addition of ammonium sulphate to 40% and 75% saturation in two subsequent steps. Samples were centrifuged at 18,500 × g for 30 min at 4 °C after each step. Consequently, the pellets were resuspended in 2.5 mL 200 mM Tris/HCl buffer with 2 mM $MgCl_2$ and 1 mM DTE, pH 7.3. Proteins were desalted by chromatography through PD 10 columns (GE Healthcare, UK) and stored at –80 °C for further use.

2.6. Enzyme assays

Enzymatic formation of glucosides was followed by incubating 100 μM OBZ with 2 mM uridine diphosphate glucose (UDPG), 1.25 mM 4-NPG, 1.25 mM salicin in 200 mM Tris/HCl buffer, pH 7.5. The reaction was started by adding 100 μL glucosyltransferase containing enzyme extract to yield a final volume of 200 μL. The incubation lasted for 60 min at 30 °C in a water bath [34]. Experiments without enzyme served as control. The reaction was stopped by precipitating the protein with 10 μL concentrated phosphoric acid. The samples were centrifuged at 13,000 × *g* for 2 min and aliquots of the supernatants were applied to LC–MS for identification of products.

Table 2
Retention times (RT) of OBZ and its metabolites and suggested identities of OBZ metabolites based on mass spectrometric data.

	RT (min)	Molecular formula	Accurate *m/z* (deviation [ppm])
OBZ	10.8	$C_{14}H_{12}O_3$	$[M+H]^+$ 229.0859
OBZ-Glu	6.9	$C_{20}H_{22}O_8$	$[M+Na]^+$ 413.1218 (2.6)
OBZ-Mal-Glu	7.3	$C_{23}H_{24}O_{11}$	$[M+Na]^+$ 499.1223 (2.1)

2.7. Enzymatic hydrolysis

Metabolite extracts prepared after SPE were freeze-dried (Speedvac, Savant Instr.) and re-dissolved in 400 μL of 50 mM sodium phosphate buffer (pH 5.0). Hydrolysis was started by incubating samples with 300 U of β-D-glucosidase (Fluka, Germany) at 36 °C for 2 h. Control experiments were free of glucosidase. Reaction was stopped by applying samples to the SPE columns. Samples were analyzed by LC–MS [33].

2.8. Alkaline hydrolysis

Plant extracts after SPE were incubated with 0.1 N sodium hydroxide for 6 h at room temperature, and control experiments were carried out in the absence of NaOH [25].

3. Results

3.1. Formation of glucose conjugate

3.1.1. Mass spectral analysis

In extracts of root material treated with OBZ, high resolution mass spectroscopy revealed a polar metabolite eluting well before the parent compound. It was identified as oxybenzone-glucoside (OBZ-Glu) with its pseudo-molecular ion at *m/z* 413.1218 $[M+Na]^+$ on TOF–MS, which corresponded to the molecular formula $C_{20}H_{22}O_8Na$ (413.1207) (Table 2). Further MS/MS experiments with the ion trap system showed that the parent compound dissociated in two major fragments of *m/z* 185 and *m/z* 251 (Fig. 1A), corresponding to the dehydroglucose and OBZ with sodium adduct during fragmentation.

3.1.2. Enzymatic hydrolysis

To confirm this glucose conjugated metabolite, samples containing the product with an *m/z* of 413 were incubated with 300 U commercially available β-D-glucosidase for 2 h. This experiment resulted in the disappearance of the signal corresponding to OBZ-Glu in LC–MS/MS (Fig. 2), while samples incubated with 50 mM sodium phosphate buffer under the same conditions still showed the peak representing OBZ-Glu.

Enzymatic synthesis

When glucosyltransferase extracts from horseradish roots were incubated with 100 μM OBZ in the presence of UDP-Glucose for 60 min, LC–MS/MS analysis revealed the same retention time and fragmentation pattern for the product formed that were previously observed in Section 3.1.1, which corresponds to OBZ-Glu.

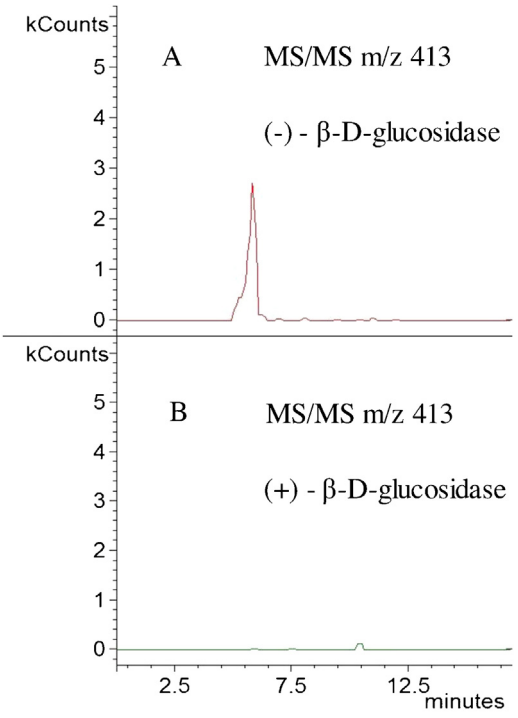


Fig. 2. LC–MS/MS chromatograms of samples containing OBZ-Glu (*SIM* mode for *m/z* 413). A untreated sample, B sample treated with β-D-glucosidase.

3.2. Identification of malonylated glucoconjugate

3.2.1. Mass spectral analysis

Another molecular ion that was detected at *m/z* 499.1223 $[M+Na]^+$, had a molecular formula confirmed by exact mass measurement on TOF–MS as $C_{23}H_{24}O_{11}Na$ (499.1221) with an error of just 2.1 ppm (Table 2). On ESI–MS, this molecular ion was 86 units larger than that of OBZ-Glu (*m/z* 413 $[M+Na]^+$). The mass difference of 86 suggests an additional malonyl group, which was also proposed by MS/MS. During ESI–MS/MS of *m/z* 499, loss of 44 Da resulted in a major fragment of *m/z* 455, and fragments of *m/z* 413 [*M*-malonyl + Na]$^+$ and *m/z* 251 [*M*-malonyl-dehydroglucose + Na]$^+$ were also generated (Fig. 1B) . The loss of CO_2 (*m/z* 44) due to the decarboxylation of malonic acid during fragmentation of a malonyl-glucopyranoside has been described in previous studies [23,35]. On this basis, our second metabolite was identified as oxybenzone-(6-*O*-malonyl)-glucoside, which was formed in a sec-

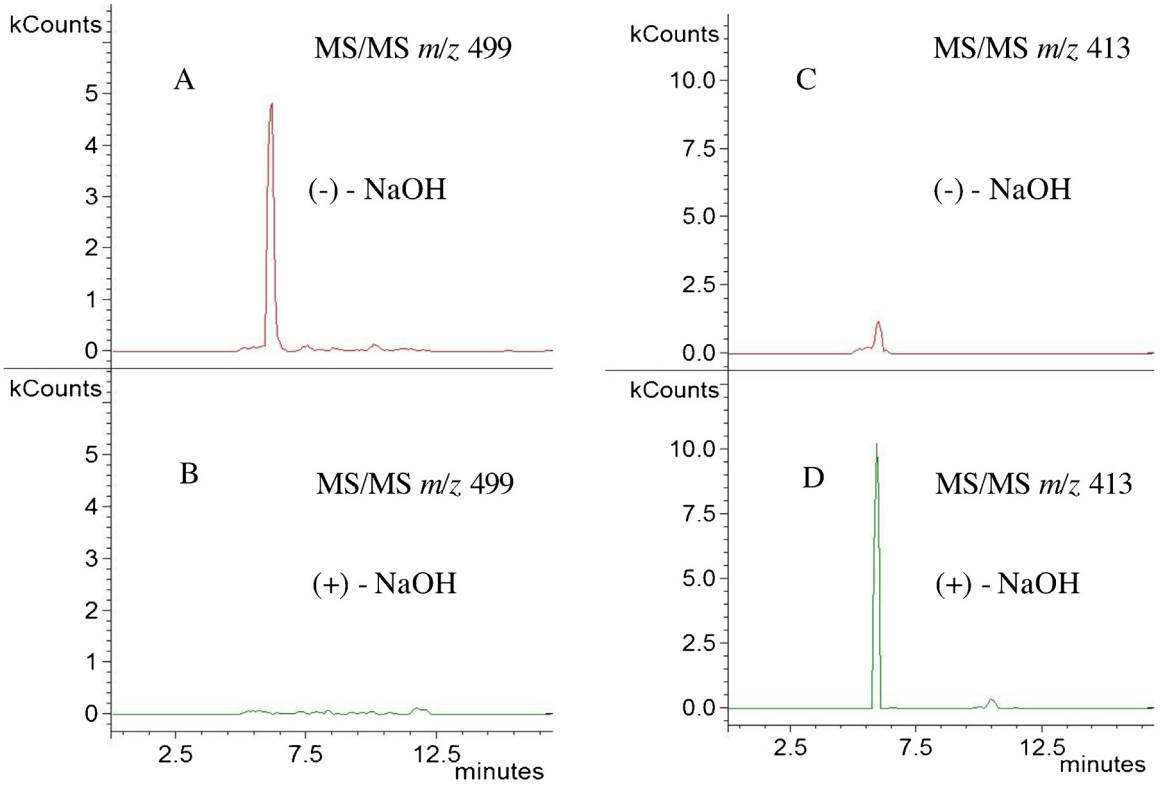


Fig. 3. LC–MS/MS chromatograms (*MRM* mode for *m/z* 499 and *m/z* 413) of samples containing OBZ-Mal-Glu and OBZ-Glu after incubation without (A, C) and with (B, D) 0.1 N NaOH for 6 h. (2-column fitting).

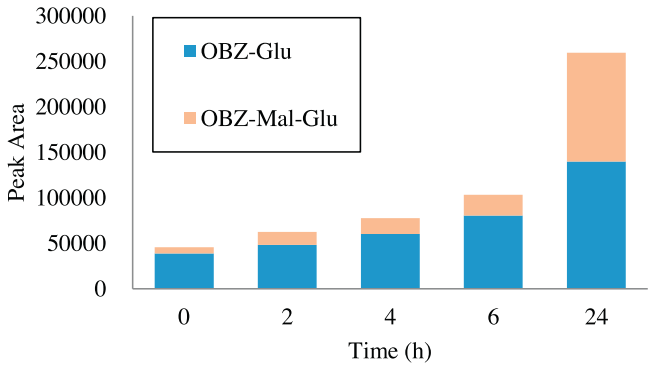


Fig. 4. Increase of OBZ-Glu and OBZ-Mal-Glu in hairy roots after 2, 4, 6 and 24 h transfer into fresh medium without oxybenzone.

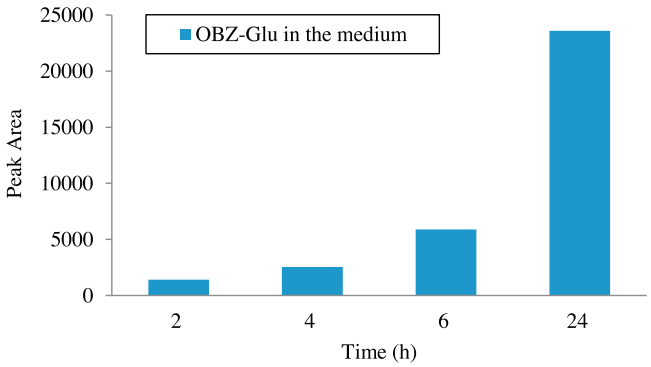


Fig. 5. Increase of OBZ-Glu in growth medium after 2, 4, 6 and 24 h transfer into fresh medium without oxybenzone.

ondary conjugation step with conjugation of malonic acid on the first metabolite.

3.2.2. *Alkaline hydrolysis*

To confirm the structure of OBZ-Mal-Glu, root extracts containing OBZ-Mal-Glu were incubated with 0.1 N NaOH at room temperature for 6 h. The NaOH completely hydrolyzed the ester bond of OBZ-Mal-Glu (Fig. 3A, B), and released free OBZ-Glu. However, the OBZ-Glu is resistant to alkaline hydrolysis, therefore, an increase of the glucoconjugate in Fig. 3C, D was observed.

3.3. *Time dependent formation of conjugated metabolites in roots*

OBZ-Glu and OBZ-Mal-Glu were formed within the first 3 h of treatment with OBZ, the OBZ-Glu was particularly dominant. The lack of reference materials of metabolites prevents the direct quantification of conjugates in plants; therefore, concentrations are given as arbitrary units (Peak area, Fig. 4). In the following hours the hairy roots were incubated in fresh medium without OBZ, but still concentrations of both metabolites increased with incubation time (Fig. 4). After 24 h, the amount of OBZ-Glu and OBZ-Mal-Glu was 3.6 times and 17.3 times higher than 0 h, respectively. Moreover, the malonylated glucoconjugate became more abundant and accounted for a higher ratio of all metabolites after 24 h, whilst at 0 h only 15% had been identified as malonyl conjugate (Fig. 4).

3.4. *Exudation of metabolite into medium*

OBZ glucopyranoside was not only found in root tissue, but also in the culture medium. After the transfer of cells into the fresh medium, OBZ-Glu released in the medium continued to increase up to 16.8 fold toward the end of the experiment compared to 2 h (Fig. 5). However, the distribution of metabolites differed from that inside the cells, since no malonylated metabolite was detected in the medium.

4. Discussion

The degradation pathway of OBZ in mammals mainly depends on hydroxylation mediated by the cytochrome P450 enzyme system [36–39] and phase II enzymes to form glucuronide or sulfate conjugates [37,40]. Accordingly, in plants, transformation of OBZ quickly occurs via direct glucosylation during phase II reactions (Fig. 6), which could be attributed to the already existing hydroxyl group of OBZ. However, no free phase I metabolites of OBZ have been detected in plants. Since plants do not possess microsomal UDP-glucuronosyltransferase [20] but glucosyltransferase instead, they use glucose as transfer partner, and hence, a glucose conjugate of OBZ was formed rapidly in the horseradish culture within just three hours of exposure. Although the stereoisomeric structure of the metabolites cannot be confirmed by mass spectrometry, the hydrolysis of OBZ-Glu by β-D-glucosidase supports that the hexose represents a β-D-glucopyranose group, which forms an *O*-glucoside. Enzymatic synthesis of OBZ-Glu with glucosyltransferase (GT) extracted from hairy roots proved the involvement of GT for glucose conjugation.

Glucosylation provides precursor molecules for further esterification with malonic acid, of which β-D-(6-*O*-malonyl)-glucoside is one of the most common products in plants [20]. Several studies have detected (6-*O*-malonyl)-glucosides of PCP, chlorophenols and triclosan in soybean, wheat, common duckweed and carrots, respectively [30,31,41]. Malonylation has been hypothesized to protect the saccharide conjugates against enzyme cleavage and to render the products ready for storage in vacuole or cell walls [31].

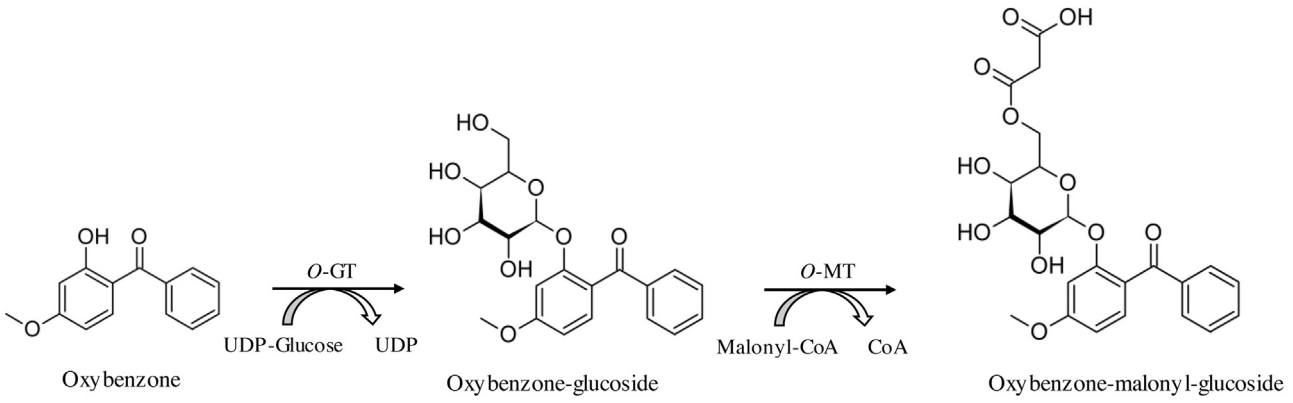


Fig. 6. Proposed metabolic pathway of Oxybenzone in *A. rusticana*.

The release of free OBZ-Glu during alkaline hydrolysis supports our idea about the further malonyl conjugation of the phase II product.

The increasing formation of two metabolites within 24 h showed that plants have a high potential to transform and detoxify OBZ. Conjugation with glucose is important in plant metabolism of xenobiotics, but glucosides might be cleaved before being indigestibly incorporated in cell walls. Secondary conjugation of glucosides with malonic acid is of special interest in this context, since malonylation may stabilize the conjugate, prevent the release of xenobiotic glucosides from plant cells and on the contrary enhance vacuolar sequestration [42]. Consistent with this theory, after 24 h incubation, only OBZ-Glu was excreted to the growth medium while leaving the malonylated metabolite inside the plants. Similar phenomena were observed in thale cress and tabacco treated with naphthols. In that case the knockout of the malonyltransferase gene (AtPMaT1) had increased the release of naphthol glucoside into media, while a force expression of AtPMaT1 decreased export and increased deposition of naphthols as malonates [29].

5. Conclusion

Our results indicate that plants may be able to take up and degrade the most common UV filter, oxybenzone. More than 20% of the initial OBZ (100 μM) was eliminated from the culture medium by hairy roots after just 3 h of exposure. Two novel metabolites were detected in the treated hairy roots and identified as OBZ-Glu and OBZ-Mal-Glu. Metabolite formation increased with incubation time. The fragmentation pattern of metabolites in MS/MS was convincing for identification. Enzymatic synthesis and hydrolysis as well as alkaline hydrolysis contributed to the characterization of metabolites.

The horseradish hairy root system provides us with a simple way to explore the metabolism of oxybenzone in plants. It is an easy-to-handle culture, and allows equal distribution of compound throughout the tissue. This study contributes to a better understanding of detoxification pathways of oxybenzone in plants. The results also suggest the use of plants for phytoremediation of UV filter compounds, and provide an appropriate alternative for treating emerging contaminants in wastewater. Research about oxybenzone metabolism in aquatic macrophytes and effects on detoxification defense enzyme systems are underway.

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Concentration effects of the UV filter Oxybenzone in *Cyperus alternifolius*: assessment of tolerance by stress related response

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Abstract

Phytoremediation has been proposed to reduce the load of the sunscreen oxybenzone (OBZ) in aquatic environment. Despite the proven removal efficiency of this compound, little is known about its influence, particularly oxidative stress on plants. In this study, a short term incubation of macrophytic *Cyperus alternifolius* was performed to prove plant´s ability to withstand the stress. Detached shoots were immersed in medium spiked with different concentrations of OBZ (50, 100 and 500 µM) for 2, 4 and 7 days, respectively. Increased formation of O_2^- and H_2O_2 in *Cyperus* treated with OBZ was characterized by intense colorization following histochemical staining. Alterations of enzyme activities involved in the antioxidative defense system indicate an adaptive response of *C. alternifolius* to this xenobiotic stress. Quantification of lipid peroxidation reveals that no significant membrane damage occurred during incubation with OBZ. Overall, 50 µM OBZ (ten-fold higher than the amount frequently detected in the environment) exhibited low toxic effects. Accordingly, this pilot study provides information on the potential use of *Cyperus* to remove emerging sunscreen contaminants from water bodies.

Keywords: Oxybenzone; ROS; H_2O_2; antioxidative enzymes; MDA; GST

1. Introduction

UV filters are active ingredients in many personal care products. Consumption of UV filters increased with growing awareness of sunburn or danger of skin cancer caused by excessive UV radiation. On the other hand, release of UV filters during human aquatic activities or insufficient removal of UV filters from wastewater by conventional treatment plants lead to increasing occurrence of these compounds in aquatic environment. As a consequence, negative effects caused by UV filters such as altered hormonal activities in the aquatic organisms have been reported (Fent et al. 2010). Oxybenzone (OBZ) is one of the frequently applied UV filters and 70% of the non-mineral sunscreens contain this compound (Environmental Working Group 2015). It has been considered as an emerging contaminant due to its ubiquity in lakes, municipal wastewater effluent and coastal areas in concentrations up to 1.4 mg/L (Balmer et al. 2005; Fent et al. 2010; Downs et al. 2016). Accumulation of OBZ in aquatic organisms has been observed particularly in fishes and subsequent adverse effects on their endocrine system have been reported (Balmer et al. 2005; Blüthgen et al. 2012; Downs et al. 2016). Moreover, literature has demonstrated significant contribution of OBZ (≥2.28 µg/L) to the deterioration of coral reefs (Downs et al. 2016). In humans, OBZ has been detected in the urine of 96.8% of U.S. residents (Calafat et al. 2008) and lower birth weight in baby girls was reported for mothers frequently exposed to OBZ (Wolff et al. 2008). Current treatment techniques, including preliminary, primary & secondary treatments, chlorination, UV disinfection, filtration over sand and activated carbon showed removal efficiencies of OBZ throughout the year in a range of 12 - 92% (Tsui et al. 2014). However, even at a removal efficiency of 92.7%, OBZ has still been detected in the effluent at concentration of 153 ± 121 ng/L (Liu et al. 2012).

In light of the increasing concerns, there is a need for better treatment solutions. Due to the inefficiency of conventional processes, phyto-treatment has been proposed to cope with pharmaceuticals and personal care products (Pilon-Smits 2005; Schröder et al. 2007). Vegetation is at the heart of this green approach, and to know whether contaminants to be treated are toxic to plants is therefore an issue of high priority which needs to be considered to improve plants of biotechnological interest. Many xenobiotics have been

shown to exert phytotoxic or even lethal effects on plants. For example, plant biomass of *Phragmites australis* has been inhibited significantly by 92% and 40% upon application of cadmium and pentachlorophenol (Hechmi et al. 2014). High concentrations of the antibiotics ciprofloxacin, oxytetracycline and sulfamethazine have posed toxic effect on the root activity and leaf chlorophyll of the same plant species (Liu et al. 2013). Exposure to metformin, ciprofloxacin and narasin has led to negative effects on the growth and development of carrots (Eggen et al. 2011). As one mode of action, xenobiotics trigger active formation of reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH^-), etc. Excessive accumulation of ROS results in cellular damages such as membrane lipid peroxidation which generates a variety of products, some of these react with protein and DNA and as a result are toxic and mutagenic (Marnett 1999). Malondialdehyde (MDA) is a product of lipid peroxidation (Mittler 2002). A significant increase of MDA was detected in roots of alfalfa plants treated with sulfamethoxazole, diclofenac and 17α-ethinylestradiol individually and in combination, a doubled increase was observed in the roots after combined exposure compared to the individual treatment, indicating higher rates of lipid peroxidation and accumulative effect of xenobiotics (Christou et al. 2016).

On the other hand ROS serve as signals to activate stress response, enhancing antioxidative mechanisms to control intracellular ROS concentrations. Involvement of plant antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7) and enzymes of the ascorbate-glutathione cycle has been reported to protect treated tissues from oxidative stress. For example, SOD, CAT and POX activities increased generally in leaves of *Typha* spp. exposed to carbamazepine (Dordio et al. 2011). Additionally, glutathione *S*-transferases (GST, EC 2.5.1.18) as dominant detoxification enzymes also contribute to the metabolism of oxidative stress products (Schröder et al. 2003). Fronds of *Azolla* spp. applied with pretilachlor exhibited increased activities of GST to remove toxic and reactive intermediate products of lipids and proteins (Prasad et al. 2016).So far, information regarding the plant´s ability to cope with OBZ has focused on uptake and transformation (Chen et al. 2016), whereas understanding of plant tolerance mechanisms is insufficient. To date, knowledge

of oxidative stress caused by OBZ has been demonstrated in human skin and zebrafish eluthero-embryos (Hanson et al. 2006; Blüthgen et al. 2012). Therefore, the aim of this study was to evaluate the phytotoxicity of OBZ in the fast-growing macrophyte *Cyperus. alternifolius*. *C. alternifolius* has been widely used in constructed and natural wetlands located in subtropical and tropical areas, and it was selected for this study due to its great potential to tolerate environmental stress (Hadad et al. 2006; Mishra et al. 2015). Changes of H_2O_2 and O_2^- in leaves exposed to different levels of OBZ were particularly measured to quantify the ROS level elevated by different OBZ concentrations. Involvement of key antioxidative enzymes in the mitigation of OBZ-induced oxidative stress in leaves was also identified to evaluate the tolerant capacity of *Cyperus* with respect to OBZ.

2. Materials and methods

2.1. Experiment setup

An assay with detached leaves was developed for rapid determination of plant response to stress (Pettitt et al. 2011). For this purpose, young *Cyperus* shoots were exposed to OBZ concentrations higher than detected in real environment to ensure sufficient uptake by the plants.

For the experiments, whole plants had been grown in modified Hoagland solution (Fediuc and Erdei 2002) with following composition: 0.4 mM Mg^{2+}, Ca^{2+}, K^+, SO_4^{2-} and NO_3^-, 0.1 mM $H_2PO_4^-$, 2 µM H_3BO_3 and Fe^{3+}, 0.2 µM Mn^{2+}, 0.1 µM Zn^{2+} and Cu^{2+}, 0.02 µM MoO_4^{2-}. All chemicals used were analytical grade.

Bundles of young *Cyperus* shoots with fully developed leaf whirls were cut under water, immediately immersed in falcon tubes filled with Hoagland solution and spiked with OBZ at each concentration (50, 100 and 500 µM) for three exposure periods (2, 4 and 7 days). Shoots without OBZ treatment (control) were established in parallel. Triplicate falcon tubes were set up for each concentration and exposure period; each containing four *Cyperus* shoots of homogenous size. The experiment was conducted in a greenhouse with 12/12h light/dark cycle at 20/15 °C. Leaf and stem samples from each treatment were harvested at corresponding times and frozen in liquid nitrogen immediately after the

sample fresh weight was determined.

2.2. Estimation of O_2^- and H_2O_2 content

O_2^- can be detected through the reduction of nitro blue tetrazolium chloride (NBT) to blue, water insoluble formazan (Brandes and Janiszewski 2005). The product was detected based on the method described by Obermeier and co-workers with slight modifications (Obermeier et al. 2015). *Cyperus* leaves were incubated in the dark with 5 mL 0.25 mg/mL NBT in 25 mM HEPES/KOH buffer (pH 7.6) for 2 h.

The detection of H_2O_2 was performed by staining with 3,3'-diaminobenzidine (DAB), and the manifested brown coloration of the leaves was due to the oxidation and precipitation of DAB by H_2O_2 (Cheng 2012). The method of Cheng (2012) was adjusted to achieve the best staining results in our experiment. Leaf samples were incubated in the dark with 0.25 mg/mL DAB in 50 mM Tris/Acetate buffer (pH 5.0) for 24 h.

In both cases, samples were subsequently boiled in 95% ethanol for 30 min to remove chlorophylls. The staining results were then recorded using a stereomicroscope (Leica M125) equipped with an HD camera (Leica MC120). To classify and visualize the intensity of staining, a GIS-software (IDRISI Selva) was used to further analyze the images.

2.3. Preparation of crude enzyme extract

The extraction of antioxidative enzymes was carried out according to the procedure described by Schröder and co-workers (Schröder et al. 2005). Two grams of frozen leaves were powdered under liquid nitrogen and homogenized in 100 mM Tris/HCL buffer, pH 7.8, containing 5 mM EDTA, 1% PVP K90, 5 mM dithioerythritol, 1% nonidet P40, and samples were centrifuged for 30 min at 20,000 rpm and 4 °C. Proteins in the supernatant were precipitated by addition of ammonium sulfate to 40% and 80% saturation, respectively. The extracts were centrifuged at 20,000 rpm after each step, and the resulting pellets were resuspended in 2.5 mL of 25 mM Tris/HCl buffer (pH 7.8) and consequently desalted with PD 10 columns (GE Healthcare, UK).

2.4. Enzyme assays

Enzyme activities were determined with a 96-well spectrophotometer (Spectra max Plus 384, Molecular devices, Germany). Protein content was quantified by the method of Bradford (1976) with bovine serum albumin as a standard protein. The specific activity is expressed as the enzymatic formation or consumption of one mole product per second [katal] in the enzyme extracts.

SOD activity was determined by its deceleration of adrenchrome formation in the autoxidation of epinephrine propagated by O_2^-. The assay contained: 158 µL 62.5 mM Na_2CO_3/$NaHCO_3$ buffer, pH 10.4, 2 µL 1.3 U/mL catalase, 20 µL epinephrine (25 mg in 30 mL 0.1 N HCL) and 20 µL enzyme extracts. Assays without enzyme served as control, and the activity was recorded at 480 nm for 5 min. One unit of SOD activity is defined as the amount of protein required for 50% inhibition of adrenochrome formation in controls (Polle et al. 1989).

CAT activity was measured by the decomposition of hydrogen peroxide at 240 nm (ε = 0.036 $mM^{-1}cm^{-1}$), the reaction mixture consists of 100 µL 100 mM KH_2PO_4, pH 7.0 , 40 µL 200 mM H_2O_2 and 10 µL enzyme samples (Verma and Dubey 2003).

POX activity was followed by the oxidation of guajacol to tetraguajacol in the presence of H_2O_2 at 420 nm (ε = 26.6 $mM^{-1}cm^{-1}$). The reaction mixture consisted of 3.4 mM guajacol and 0.9 mM H_2O_2 dissolved in 50 mM Tris/HCl buffer, pH 6.0 (Diekmann et al. 2004).

Ascorbate peroxidase (APOX, E.C. 1.11.1.11) activity was analyzed according to the method described by Lyubenova and co-workers with modifications (2015). Each reaction contained 40 µL 5 mM ascorbate, 20 µL 1 mM H_2O_2, 20 µL 1 mM EDTA and 20 µL enzyme extract in 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) in a final volume of 200 µL, and the activity was calculated by the oxidation of ascorbate at 290 nm (ε 2.8 $mM^{-1}cm^{-1}$).

Glutathione reductase (GR, EC 1.6.4.2) was assayed in 100 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA, 10 mM GSSG, 20 mM NADPH and extracted protein with significant modifications (Vanacker et al. 1998). Its activity was evaluated by the extinction in absorbance at 340 nm (ε = 6.2 $mM^{-1}cm^{-1}$) resulting from the consumption of NADPH.

GST activity was determined with the model substrates 1-chloro-2,4-dinitrobenzene (CDNB), fluorodifen and p-nitrophenylacetate (pNpa) following published standard methods (Habig et al. 1974; Schröder et al. 2008). In the assays, enzyme extracts were incubated with 1 mM reduced glutathione (GSH), 1 mM substrate and 100 mM buffer (CDNB: Tris/HCl buffer pH 6.4; fluorodifen: Tris/HCl buffer pH 7.5; pNpa: potassium phosphate buffer pH 7.0). The formation of GSH conjugates with substrate was recorded at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) for CDNB, at 400 nm (ε = 17.2 $mM^{-1}cm^{-1}$) for fluorodifen and at 400 nm (ε = 8.79 $mM^{-1}cm^{-1}$) for pNpa, respectively.

2.5. Histochemical detection of malondialdehyde

Lipid peroxidation levels in *Cyperus* tissues were estimated indirectly in terms of MDA content, by measuring thiobarbituric acid reactive species (TBARS) according to the methods of Heath& Packer (1967) with modifications. 0.5 g of plant materials were grinded under liquid nitrogen, and diluted in 5 mL 0.1% trichloroacetic acid (TCA). The samples were centrifuged for 5 min at 10,000×g, 5 mL supernatant were collected and mixed with 2 mL 0.5% thiobarbituric acid (TBA) diluted in 20% TCA. Subsequently, the samples were incubated in a water bath at 95°C for 30 min. The reaction was stopped by incubation on ice, filtered by Miracloth (Calbiochem) and centrifuged again at 10,000 × g for 5 min. The fluorescent product was determined spectrophotometrically at 532 nm corrected for nonspecific turbidity by subtracting the absorbance at 600 nm (Spectra MAX 190 J, Molecular Devices). MDA equivalents were calculated as µmol TBARS per gram fresh weight by using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.6. Data analysis

Statistical analyses were performed with the software GraphPad Prism v5.0. Two-way analysis of variance (ANOVA) with Bonferroni post-test was applied to determine significant differences between groups, and comparisons were considered significantly different for $p < 0.05$.

3. Results and discussion

3.1. Plant growth

Since detached shoots and leaves continue to expand, relative growth rates (RGR) were monitored to evaluate OBZ toxicity towards *Cyperus* at different OBZ concentrations (Fig. 1). After 2 d incubation, RGR of exposed plants were significantly lower than those of control plants, except for those in the 50 μM treatment which had RGR similar to control. Subsequently after 4 d incubation, decrease of biomass occurred in all exposed plants, and RGR of *Cyperus* exposed to 100 and 500 μM were significantly lower than those of controls and 50 μM exposure. At the end of the experiment, all exposed plants showed decreased biomass compared to t=0. These results imply that OBZ seems to affect plant development rapidly, and this effect is strongly dependent on OBZ concentrations. Elevated concentrations of xenobiotics are known to disturb plant growth. Bartha et al. (2010) found that bleaching in leaves of *Brassica juncea* occurred after 72 h exposure to 1 mM acetaminophen and necroses were observed after 168 h of treatment. Similarly, exposure to high levels of carbamazepine (> 4.23 μM) led to a 50% reduction in total biomass weight of cucumber (Shenker et al. 2011). In the present study, high OBZ concentration (500 μM) caused considerable inhibition of shoot growth by 37% decrease of *Cyperus* fresh biomass. Therefore, in comparison to the treatment with 100 and 500 μM OBZ, it could be assumed that concentrations below 50 μM OBZ do not pose significant effects on *Cyperus* growth and performance.

3.2. ROS content

Highly reactive free oxygen radicals (O_2^-) and the stable ‘diffusible’ non-radical oxidants (H_2O_2) have been the main investigative foci of ROS biology in recent years (Gough and Cotter 2011). To evaluate the effect of OBZ on the ROS level in *Cyperus* leaves, O_2^- and H_2O_2 contents were visualized and characterized by the staining intensity.

3.2.1. Characterization of O_2^- formation and SOD activity

False coloration of formazan deposits (Fig. 2) identifies the regions of O_2^- formation in leaf tissue. Control leaves showed the smallest stained area which indicates low O_2^- formation in normal metabolic condition. Contrary to this, significant increase of O_2^- in response to

OBZ was observed in all treated shoots. In addition, the level of O_2^- in *Cyperus* increased following exposure time to OBZ stress. After 2 d exposure, 1%, 3.5% and 3% of the area was classified as containing high amounts of O_2^- (class 1-2) when exposed to OBZ at 50, 100 and 500 μM, respectively. In the following 4 d exposure, an even higher amount of O_2^- was detected compared to those observed on 2 d for each OBZ concentration. Finally, at the end of the experiment, the stained area in 500 μM OBZ treatment had increased evidently by 2.14-fold as compared to controls and accounted for 10% of the total leaf area, among which the most pronounced O^{2-} concentration accumulated in the area of the central leaf vein.

Formation of O_2^- in plants with respect to exposure to xenobiotics has been reported previously. For example, in *Lemna* fronds treated with 2.5 μg/L pethoxamide for 7 d, NBT staining areas representing higher amounts of O_2^- occupied 30% of the total area (Obermeier et al. 2015). Also, accumulation of O_2^- content following NaCl stress was noticed in duckweed, where SOD appeared to be induced to catalyze the conversion of O_2^- to H_2O_2 (Chang et al. 2012). In the present study, the change of SOD activity confirmed O_2^- accumulation (Fig. 3). Its activity was elevated when there was excessive O_2^- staining. For example, in the presence of 100 μM OBZ for 7 d, SOD activity increased 1.7-fold by effect of O_2^- overproduction (staining area increased 2.1-fold) compared to that in the same treatment after 2 d. Stimulated SOD activity has also been observed in *Oryza sativa*, *Pennisetum americanum* and *Medicago sativa* during exposure to lead, atrazine and 17α-ethinylestradiol, respectively (Verma and Dubey 2003; Jiang et al. 2015; Christou et al. 2016). On the other hand, SOD activity increased slightly in response to 500 μM OBZ over the experimental period, while after 4 d OBZ application its activity was even 32% below the control level, suggesting that high doses of OBZ could weaken the ability of *Cyperus* to remove the excessive ROS. Similarly, low SOD activity was found in roots of *P. americanum* at high concentrations of atrazine (100 mg/kg, 200 mg/kg) (Jiang et al. 2015). Thus, it is reasonable to assume that elevated SOD as a primary scavenger supports *Cyperus* to reduce O_2^- induced by low OBZ concentrations while high concentrations lead to exhaustion of the defense system.

3.2.2. Production of H_2O_2

Figure 4 presents the distribution of H_2O_2 in *Cyperus* leaves after infiltration with DAB. Images acquired after OBZ treatment displayed an increase in the stained area compared to that in controls. In general, the stained regions continued to increase over the entire exposure period. After 2 d incubation untreated leaves displayed only small and scattered stained areas. The total staining area after 50 μM OBZ treatment remained constant throughout the experiment time and the staining pattern was similar to that of the control plants, whereas the dominant staining class shifted from class 3 to class 1 over exposure period, referring to an increased production of H_2O_2 (Fig. 4). Treatment with 100 μM OBZ for 2 d led to a 4.6-fold increase of the stained area compared to control, but the increase slowed down in the following days and finally the percentage of stained area reached 6% which is 1.68-fold above the corresponding values of controls. High intensity of DAB staining was pronounced after treatment with 500 μM OBZ for 4 d and more than 60% of its stained area was classified as class 1. The total staining area from this treatment expanded during 7 d exposure, but class 2 staining area became more pronounced and accounted for 85% of the total stained area, which implies a decrease in formation of H_2O_2 as the experimental exposure continues. Besides the numerous scattered stained spots, the majority of heavy staining on the leaf exposed to higher OBZ concentrations was again recorded adjacent to the venal tissue, indicating that the intrinsic signal which stimulates the production of ROS proliferated along the leaf veins.

Increased H_2O_2 concentration has been reported in several plant species under abiotic stress. A 1.3-fold increase of H_2O_2 content in *Festuca arundinacea* exposed to hydrocarbons has been observed, however the amount of H_2O_2 has been controlled when the roots are associated with endophytic fungus (Mendarte-Alquisira et al. 2016). In this context, our results implied that high OBZ concentrations (100, 500 μM) in general caused increasing formation of H_2O_2 in leaves. Corresponding to our findings, Prasad and co-workers (2016) demonstrated that the level of H_2O_2 in *Azolla* spp. treated with pretilachlor increased with rising concentrations of this herbicide. The enhancement of H_2O_2 was ascribed to the inhibition of photosynthetic electron transport chain activity, as well as to the degradation of pretilachlor during which the ROS was produced. H_2O_2

content has also been reported to be enhanced with prolonged incubation time, e.g. in greater duckweed H_2O_2 was accumulated by 1.3-fold and 2.17-fold after application with 200 mM NaCl for 6 and 12 days, respectively (Chang et al. 2012).

3.3. Activity of antioxidative enzymes

CAT, APOX and POX are important antioxidative enzymes involved in conversion of H_2O_2 to water (Mittler 2002). GR functions together with APOX in the ascorbate-glutathione cycle, where it mediates the regeneration of GSSG to GSH (Rao and Reddy 2008). To investigate alterations in the activities of the above mentioned enzymes (including SOD), their activities were plotted relative to controls in Fig. 5, while data with statistical analysis are shown in Fig. S1. Within 2 d incubation, enzyme activities in *Cyperus* were generally stimulated (Fig. 5) except for APOX at 50 μM OBZ (Fig. S2). Activities recorded for CAT (OBZ≥50 μM), POX & GR (OBZ≥100 μM) and APOX (500 μM OBZ) increased significantly to more than 127% of control. After 4 d treatment, activities of these enzymes had returned to control level except for GR at 500 μM OBZ treatment, the activity of which was still significantly higher than that in controls. Activities of POX at 100 μM and APOX at 500 μM were found to be significantly lower than those in the corresponding controls. Samples from day 7 showed that apart from CAT (50, 100 μM) and SOD (100 μM), there was no significant difference between enzyme activities from OBZ treated and control plants. As a consequence, the enhancement of enzyme activities towards OBZ occurred transiently during the initial stage of assays, and higher induction appeared more frequently at higher concentrations. Compared to the other enzymes, CAT with an increase of 3.4 to 5.4-fold of control seems to be more active against stress caused by OBZ. This finding is supported by the conclusion of Mittler (2002) that unlike APOX, activity of CAT may not be negatively influenced and is insensitive to the redox status as it does not require reducing equivalents, the pools of which might be suppressed or exhausted during oxidative stress.

Alterations in activities of antioxidative enzymes have been demonstrated in various plant species under different types of stresses. High induction rate of CAT has been shown in plants of *Populus nigra* exposed to ibuprofen (Iori et al. 2012). Increase of CAT activity has also been reported in rice plants grown in medium containing lead, where their CAT

increased during early days to a maximum, followed by a decline (Verma and Dubey 2003). Activity of CAT in *Typha latifolia* increased after one week exposure to carbamazepine, however at the same time activity of POX was inhibited and its activity began to increase only after treatment for 21 days (Dordio et al. 2011). GR has been found to be stimulated in *Phragmites australis* under treatment with chlorocyclohexane and trichlorobenzene (Faure et al. 2012). The increased GR activity could be related to the maintaining of GSH pool which is an essential antioxidant and is partly abstracted as a substrate for GSTs, which conjugate xenobiotics and other radical products of lipid peroxidation and protein oxidation for compartmentation into vacuole (Mittler 2002; Prasad et al. 2016).

3.4. GST activity

Oxidative stress is a known inducer of plant GST, and induction has been described within few hours after contact with xenobiotics (Schröder 2001). Generally, as shown in Fig 6 and S4, *Cyperus* GST activity increased after 2 d incubation. Significant enhancement (Fig. S3) by 153.7-163.7% of controls was recorded for GST activity towards CDNB. CDNB is a traditional model substrate with high reactivity with many GST isoforms (Deponte 2013). The stimulation disappeared within 4 d for GST_{CDNB} and GST_{pNpa}, whereas conjugation with the herbicide fluorodifen was inhibited to 50-64% of control levels. On day 7, a second induction phase was noted for GST activities toward all three substrates. Significant increase was evidenced at 100 μM OBZ and this induction was stronger than the first induction detected on day-2. However, $GST_{fluorodifen}$ and GST_{pNpa} activities at 500 μM OBZ remained inhibited. This pattern resembles a second oxidative burst, as it has been described by Messner and Schröder (1999) under the influence of elicitors.

GST is speculated to be involved in the suppression of oxidative burst reactions (Marrs and Walbot 1997). Accordingly, within 2 d incubation, the enhanced activities of GST and the enzymes mentioned in section 3.3 after exposure to OBZ correspond to the elevated amount of oxidative burst. And it seems that the antioxidative enzymes in section 3.3 accounted for a major role in the defense system due to their high induction rate. However, as observed on day 7, GSTs were elevated again which refers to a second oxidative burst (as mentioned above), indicating a pivotal role of GSTs in leaf defense against OBZ stress.

Furthermore, the selective induction patterns toward different substrates suggest the presence of several isoforms of GST in *Cyperus*. Particularly those GST isoforms with affinity for CDNB are more distinctly induced during the early stage of treatment.

Although few data are available on the GST enzymes of *Cyperus*, several studies have reported the involvement of GST in the plant tolerance under different stress factors. Lyubenova and co-workers (2011) have summarized that GSTs show strong induction in rhizomes of *Typha latifolia* at high heavy metal concentrations; however, in the same report, all leaf GST activities were less than 50% of controls. Another example of high GST activities toward CDNB, fluorodifen and pNpa have been demonstrated in sunflower plants grown on zinc and cadmium contaminated soil (Nehnevajova et al. 2012). The same induction effect was observed in the roots of *F. arundinacea*, where GST_{CDNB} activity was stimulated by 63% in the presence of the hydrocarbons (Mendarte-Alquisira et al. 2016).

3.5. Lipid peroxidation

To assess the cellular damage level caused by excessive ROS, MDA concentration levels in leaf and stem were measured (Fig. 7). In stems, MDA increased slightly (6.5%) after 4 d treatment with 100 and 500 μM OBZ, and an increase of 22.2% was detected after one week incubation with 500 μM OBZ. The amounts of MDA in control and 50 μM assays were relatively stable over the whole experimental period. In leaves, increased amounts of MDA by 11.2% and 6.9% after 4 d treatments with 100 and 500 μM OBZ indicate the occurrence of lipid peroxidation. Particularly, in 500 μM OBZ incubations, the MDA content on day-7 increased 32.4% to reach a maximum of 23.44 μmol/g fresh weight (FW). In general, MDA concentrations in leaves were higher than that in stems, indicating a stronger lipid peroxidation in leaves.

Lipid peroxidation is a biochemical marker for free radical mediated injury. Altered concentration of MDA has been commonly reported in various environmental stress conditions, such as drought, salinity or metal contamination. Remarkable increase of MDA content was observed in the leaves rather than roots of pea treated with higher selenite doses (50-100 μM), showing correspondence to the increased H_2O_2 levels (Lehotai et al. 2016). Similar findings have been reported after exposure to NaCl which led to

enhancement of MDA content in greater duckweed by 1.48-fold within 6 days accompanied by 1.3-fold increased H_2O_2 content (Chang et al. 2012). Whereas MDA and H_2O_2 content in our study did not change in parallel, since the H_2O_2 amount increased significantly after 2-day incubation, the alteration of MDA concentration was delayed and the increase occurred after 4 or 7-day treatment, together with the second GST burst. This corresponds to the opinion of Halliwell & Chirico (1993) that lipid peroxidation often occurs late in the injury process. Furthermore, the change of MDA was not statistically significant in the present study, which is confirmative with the observations of Lyubenova and coworkers (2009), who reported no correlation between the uptake of heavy metals and the stress marker MDA in *Nicotiana tabacum*.

3.6. Implications for treatment of OBZ on site

Constructed wetlands have been incorporated on-site to refresh water bodies and maintain water quality. For example, the 'Bio-Park' which consists of a constructed wetland and a hydroculture system, was established directly in the Lake Fuehlingen in the city of Cologne. This lake was built near the densely populated area of Rhine River and was designed as a sports and leisure center, providing opportunity for recreational activities (Schröder et al. 2005). Such kind of lakes suffers frequently from degradation of water quality, and is particularly in a danger of UV filter (OBZ) contamination during summer. Surface waters like the pilot system 'Bio-Park' seem to be a most effective tool for improving and guaranteeing an adequate water quality in frequently used recreational lakes. Thus knowing about the tolerance of plants against sunblockers like OBZ and their removal would be extremely helpful for enhancing performance of such plant based pollution treatment.

4. Conclusions

According to the findings in the present study, the detached leaf system is a perfect model system to study short term response towards xenobiotics. Our data confirmed the oxidative stress induced by OBZ practically at higher concentrations. Overall the antioxidative enzyme activities were higher in plants exposed to OBZ than in the control, and different concentration response relationships were observed. Compared to the late and insignificant

reactions of lipid peroxidation, activities of SOD, CAT, POX and GST are sensitive parameters for characterizing the toxicity of OBZ in *Cyperus*. Given that the impact of 50 μM OBZ on *Cyperus* was similar to that of controls, and 50 μM, the lowest concentration applied in this experiment is several orders of magnitude higher than the concentration detected in the real environment, it can be expected that very limited phytotoxic effects will appear on plant systems using macrophytes like *Cyperus* for effective phytoremediation of OBZ.

Acknowledgements

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Figures:

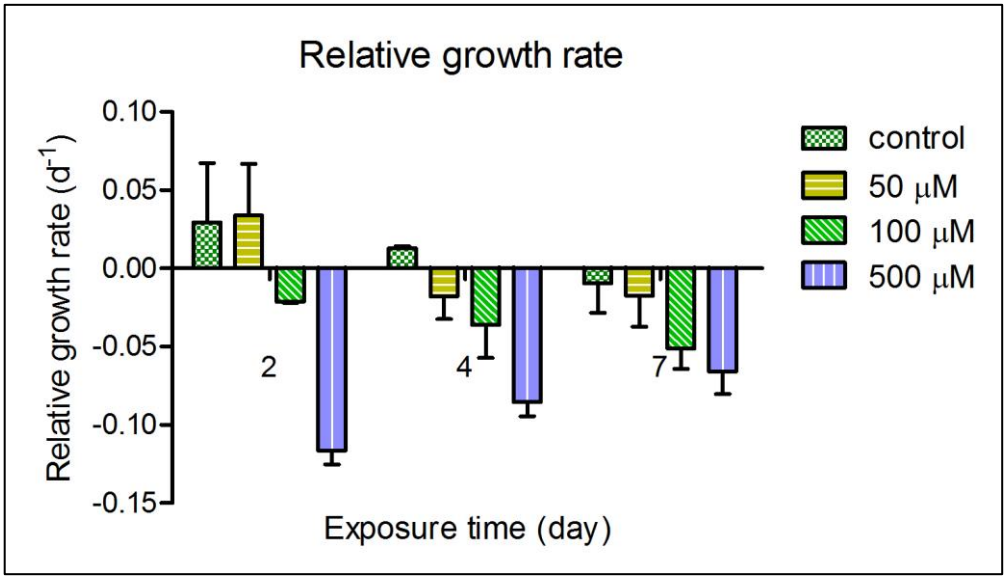

Figure 1 Relative growth rate of *Cyperus* at different times (2, 4 and 7 days) under different treatments. Data are average values from biological triplicates. The RGR was quantified by measuring the fresh weight of plants exposed to different initial OBZ concentrations at each sampling time. Relative growth rates= $(\ln W_t - \ln W_0)/t$, where W_0 is the initial plant weight and W_t is the plant weight at corresponding harvest time, t is the exposure time (day).

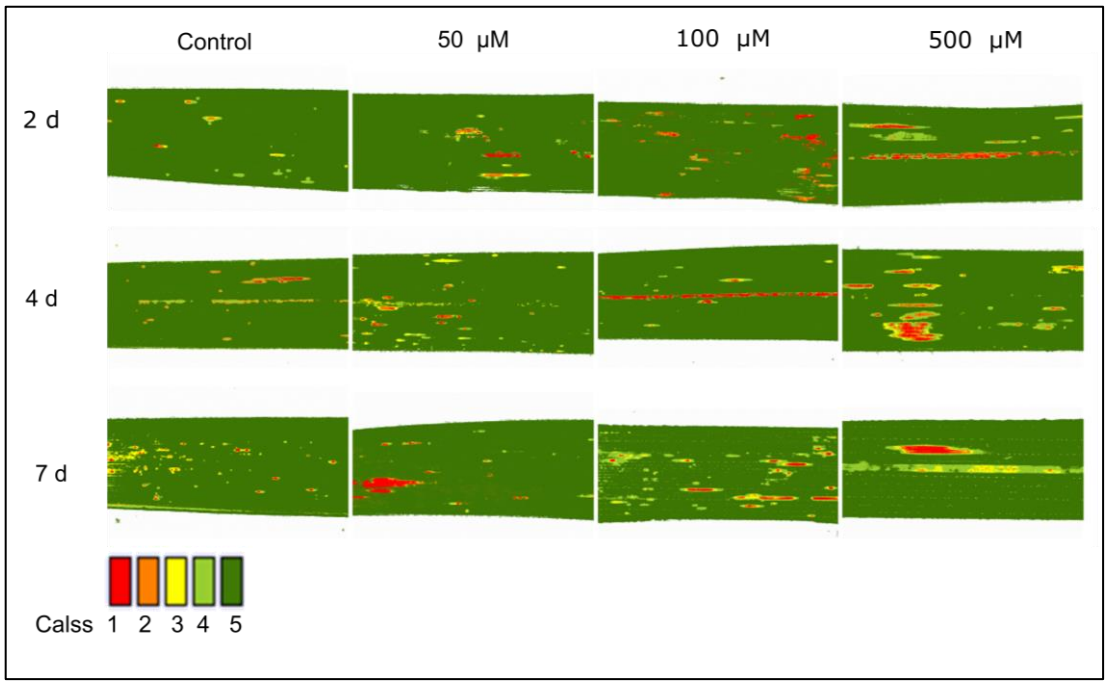

Figure 2 False coloration image of *Cyperus* leaves infiltrated with NBT. The location of formazan deposits was visualized by IDRISI and the intensity was classified by maximum-likelihood classification; class 1: high—class 5: low amount of O_2^-.

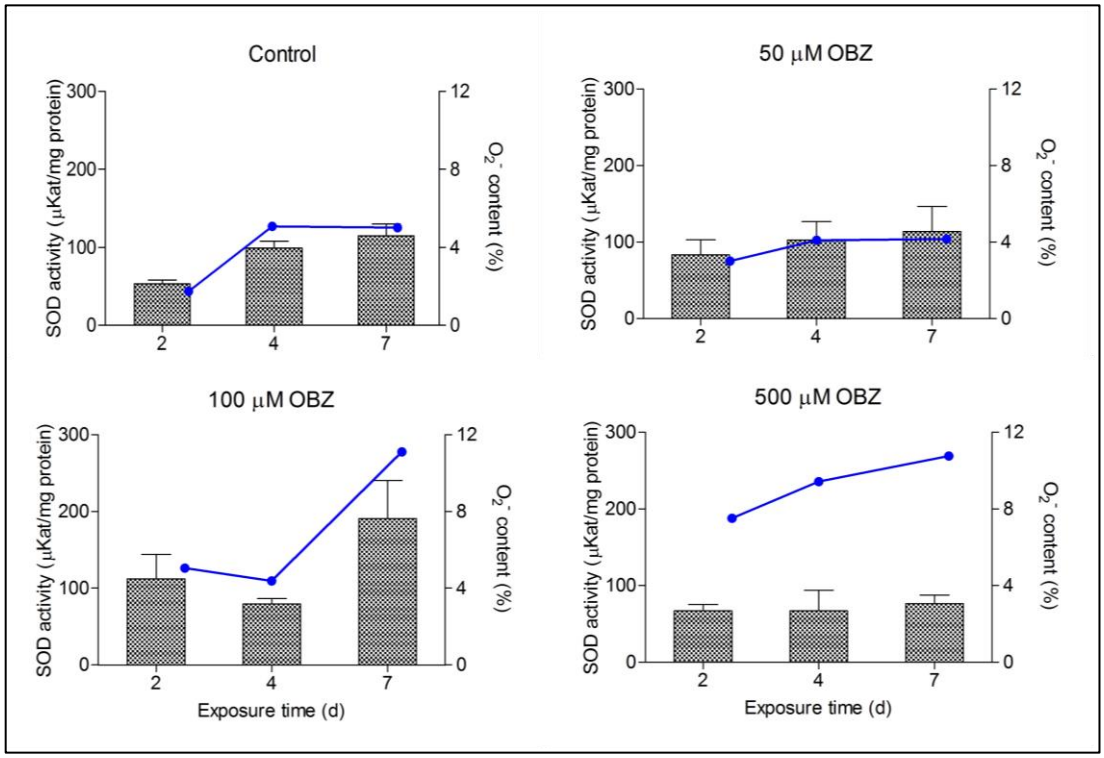

Figure 3 SOD activities (solid bars) and O_2^- content (line with dots) in *Cyperus* leaves under different treatments over exposure time. Data are average values from biological triplicates. Error bars indicate SD.

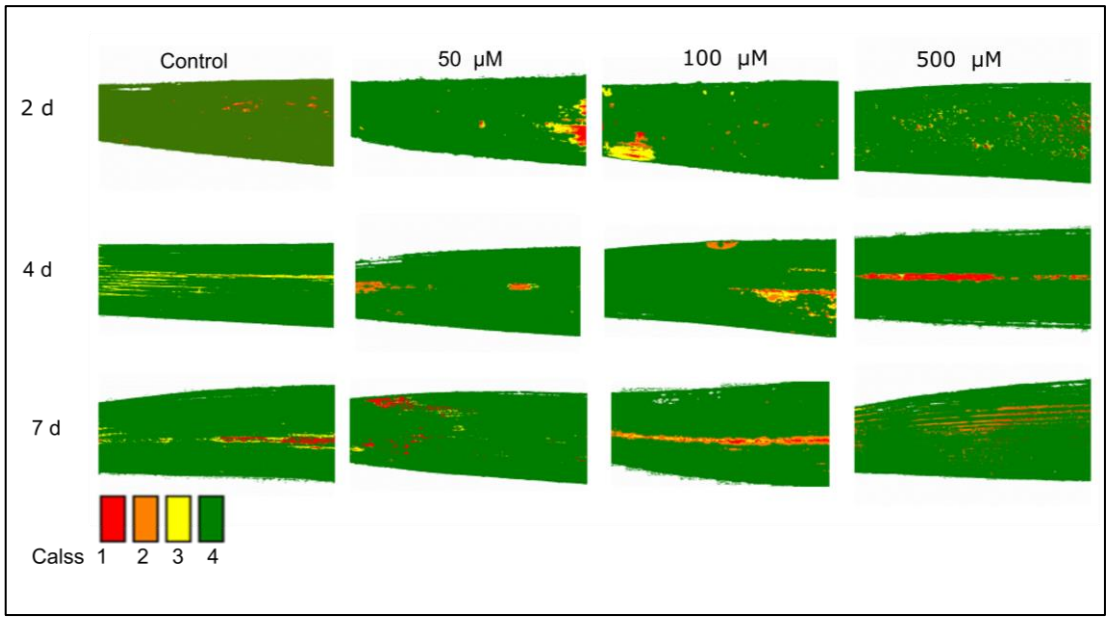

Figure 4 False coloration image of *Cyperus* leaf sections infiltrated with DAB. The location of polymerization product resulted from DAB reaction with H_2O_2 was visualized by IDRISI and the intensity was classified by maximum-likelihood classification; class 1: high—class 4: low amount of H_2O_2.

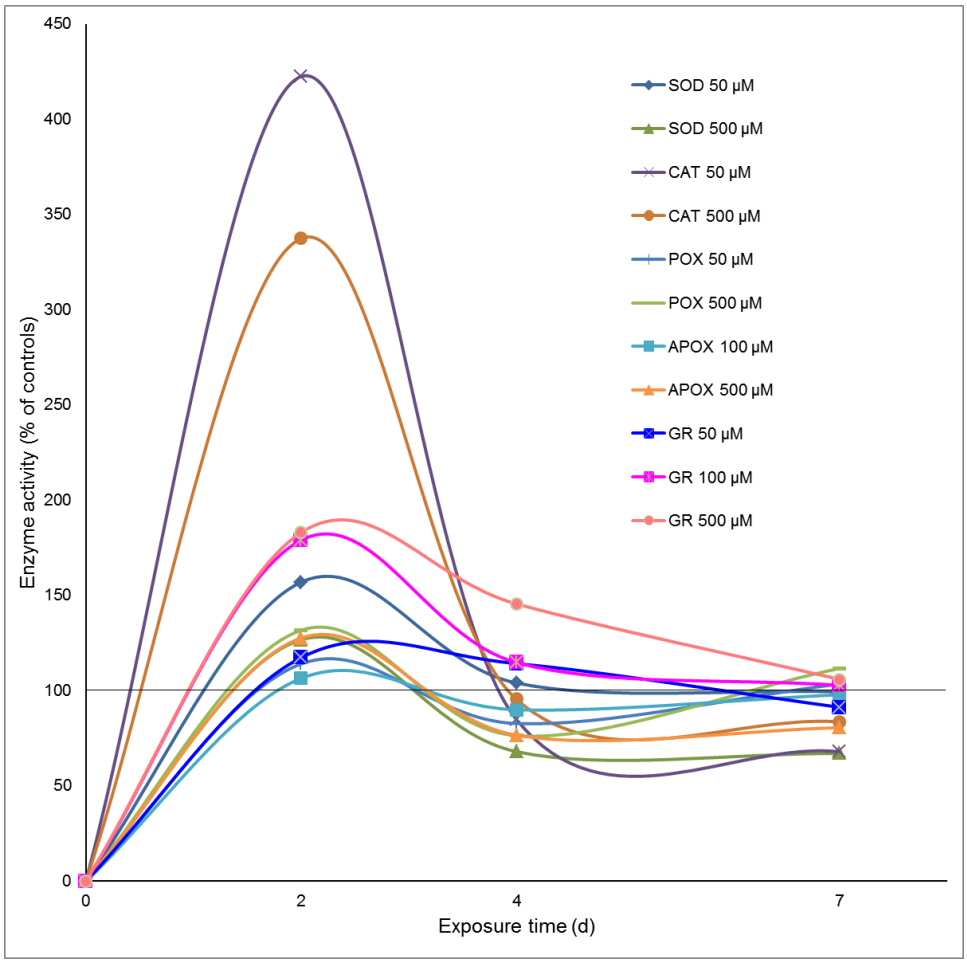

Figure 5 The effect of OBZ on enzymes involved in ROS scavenging systems. Enzyme activities are described relative to controls.

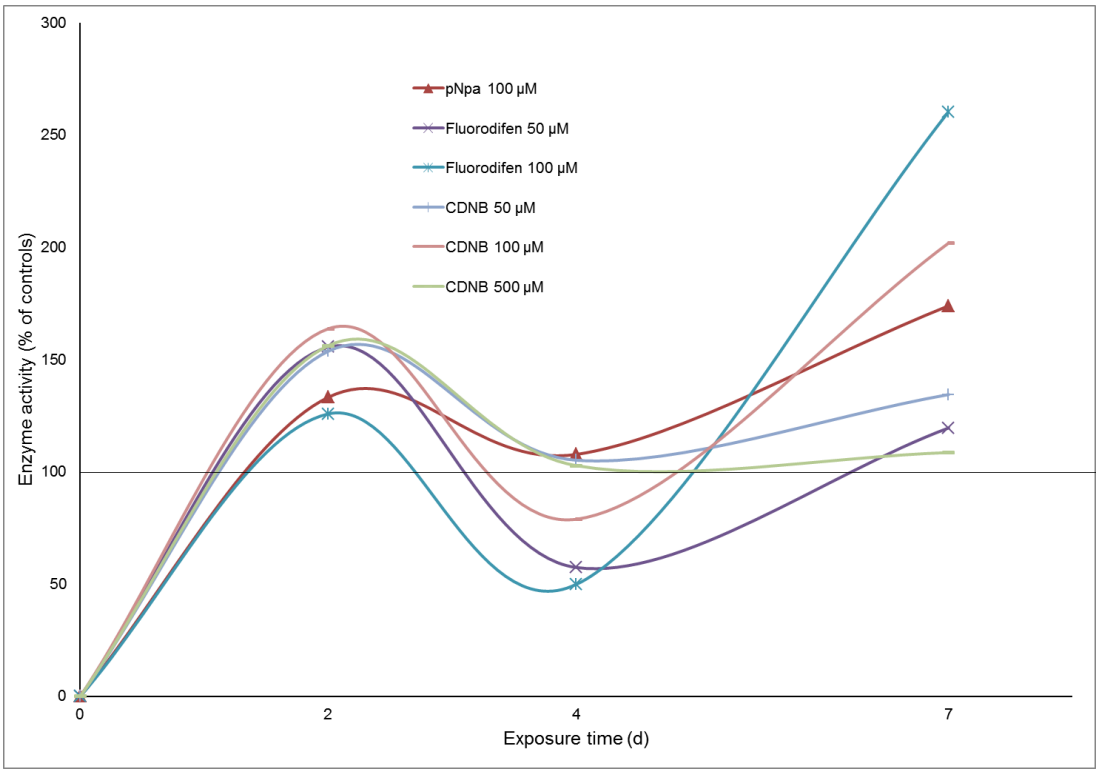

Figure 6 Biphasic inductions of GST activities toward CDNB, fluorodifen and pNpa under different OBZ concentrations. Levels of enzyme activities are depicted relative to untreated control. $GST_{Fluorodifen}$ and GST_{pNpa} at 500 µM OBZ are shown in Figure S4.

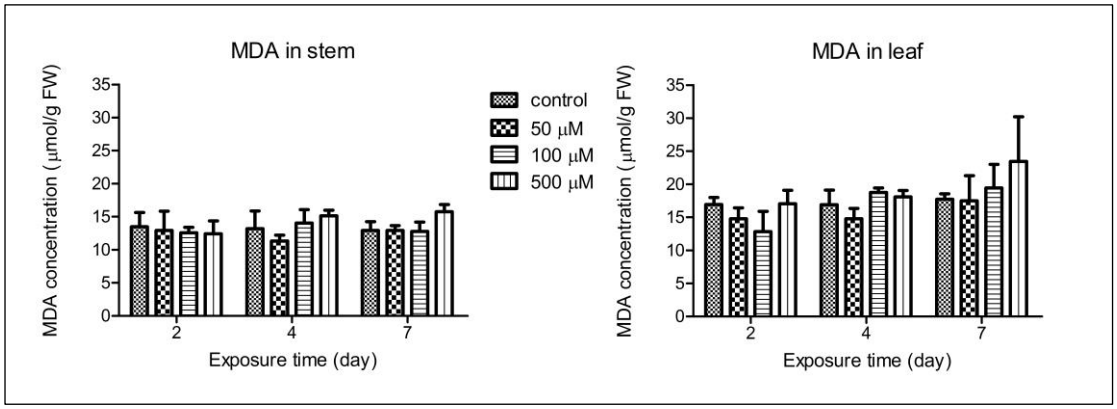

Figure 7 MDA concentrations in leaf and stem tissues at 2, 4 and 7 days under different treatments. Data are mean values of biological triplicates. Error bars indicate SD.

The following is the supplementary data related to this article:

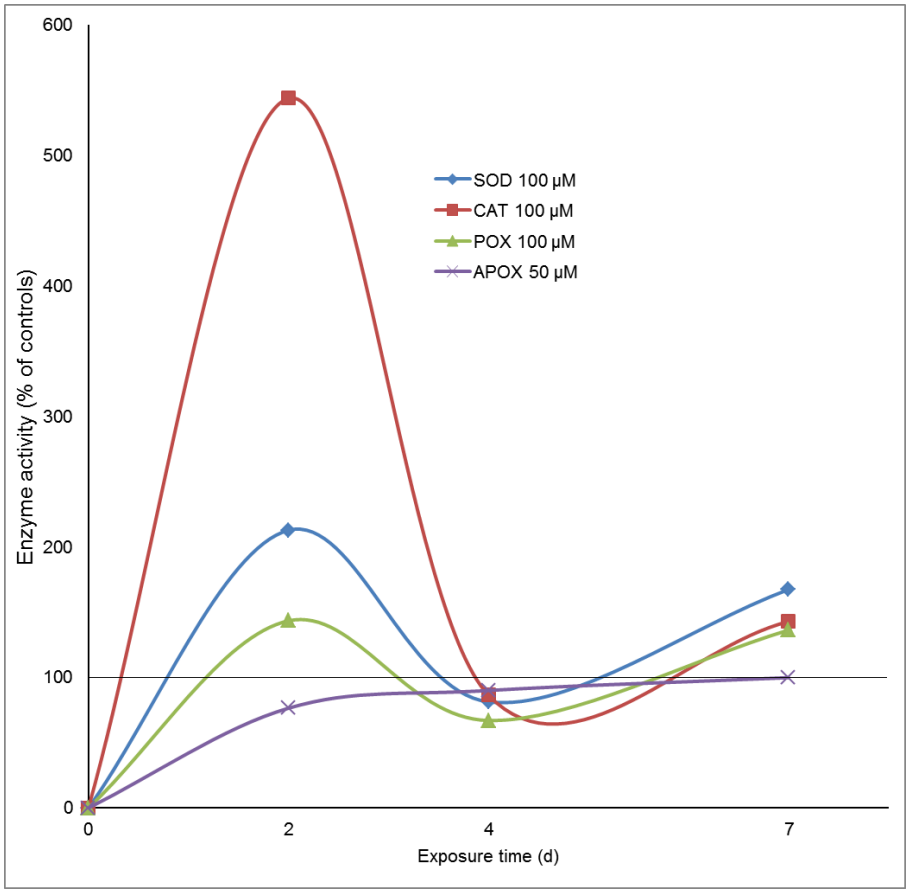

Figure S1 Effects of 50 µM OBZ on APOX, and activities of SOD, CAT and POX under 100 µM OBZ exposure. Activities are depicted relative to controls.

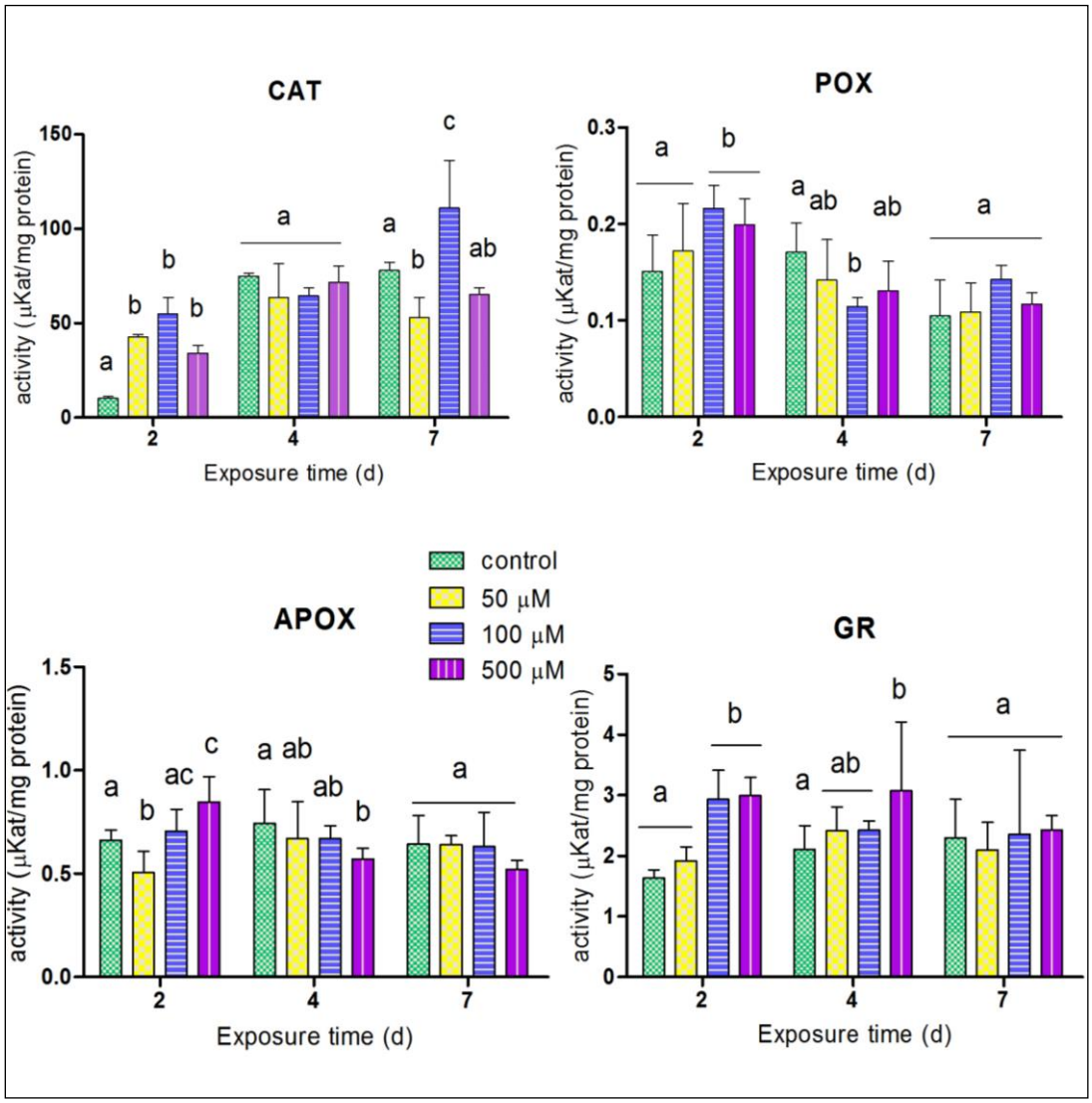

Figure S2 Distribution of antioxidative enzyme activities in leaves of Cyperus exposed to different concentrations of OBZ at each sampling time. Lowercase letters indicate significant differences according to ANOVA at $p < 0.05$. Data are means of three replicates.

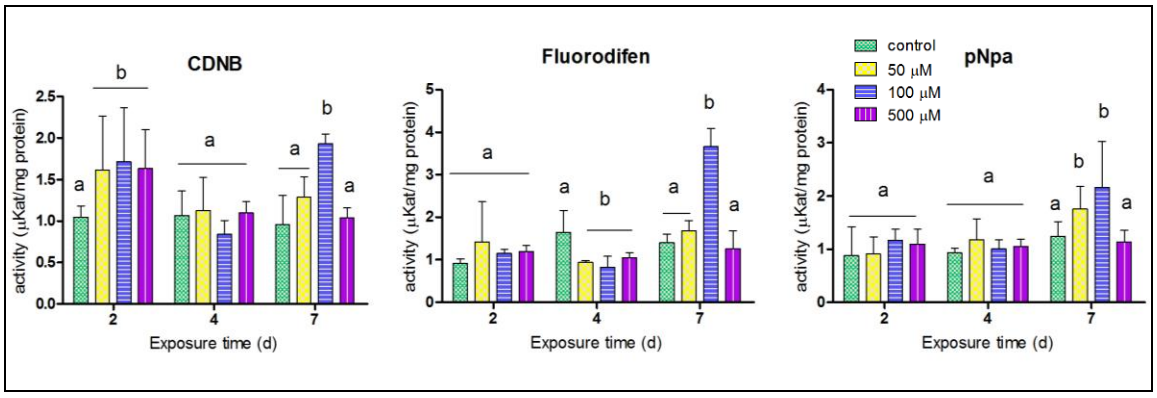

Figure S3 GST activities toward three standard substrates in leaves of Cyperus exposed to different concentrations of OBZ at each sampling time. Lowercase letters indicate significant differences among different concentrations according to ANOVA at $p < 0.05$. Error bars indicate SD (n=3).

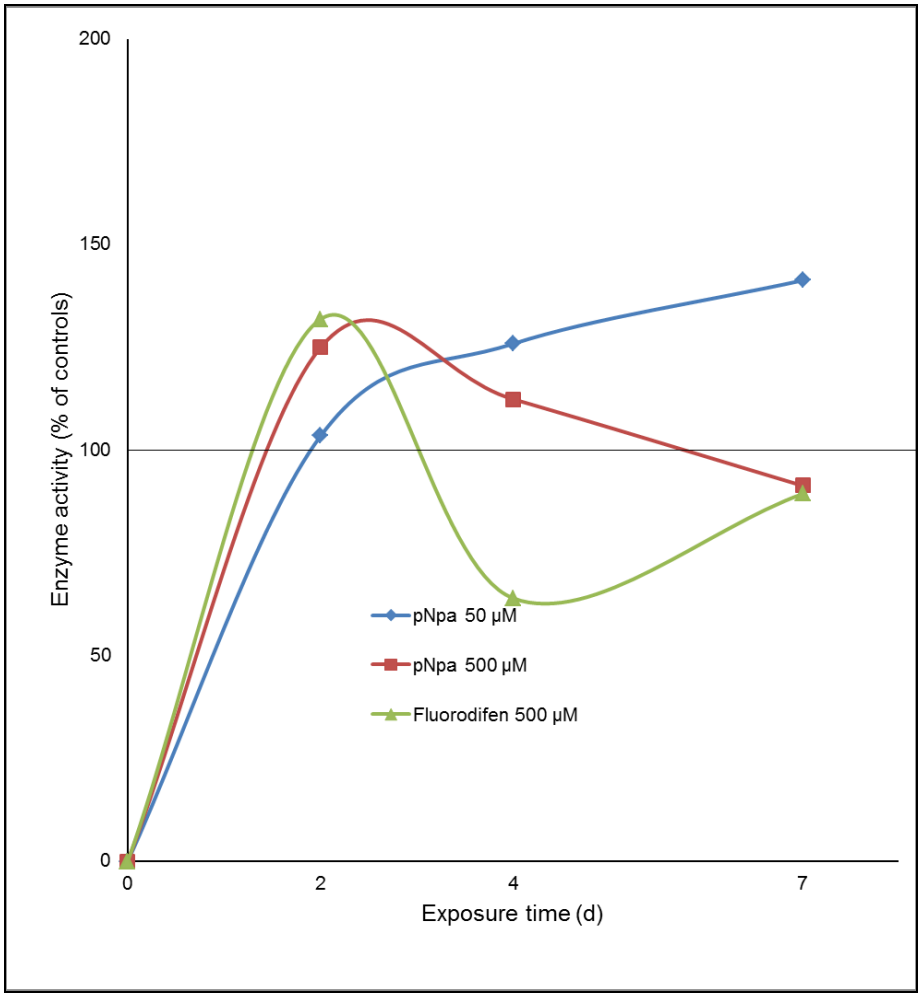

Figure S4 Effects of 50 μM OBZ on GST_{pNpa}, and activities of GST_{pNpa} and $GST_{fluorodifen}$ under 500 μM OBZ exposure. Activities are depicted relative to controls.

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Fate of the sunscreen compound oxybenzone in *Cyperus alternifolius* based hydroponic culture: Uptake, biotransformation and phytotoxicity

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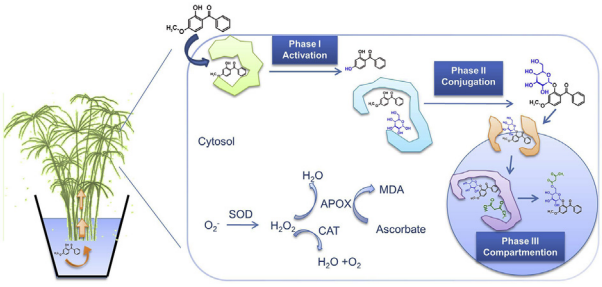
HIGHLIGHTS

- The sunscreen oxybenzone is suspected to be an endocrine disruptor.
- Oxybenzone can be removed from hydroponic medium by *C. alternifolius*.
- Higher accumulation of oxybenzone occurs in roots rather than in shoots.
- 2,4-dihydroxybenzophenone as precursor of further metabolites is for the first time detected *in planta*.
- Plant defense systems are activated to overcome the stress caused by oxybenzone.

GRAPHICAL ABSTRACT



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Stress response

ABSTRACT

Oxybenzone (OBZ), a common ingredient in sunscreens and personal care products, has been frequently detected in effluents from municipal wastewater treatment plants and also in surface waters. OBZ is an emerging contaminant due to its adverse impacts on marine/aquatic ecosystems. To investigate the removal and degradation capacity of phytotreatment for OBZ, the common wetland plant species *Cyperus alternifolius* L. was exposed to this compound at 5, 25 and 50 μM for 120 h, respectively. Continuous uptake by roots and accumulation in plant tissues was observed over the exposure time, and depletion of spiked OBZ from the aqueous medium exceeded 73.9 ± 9.1% after 120 h. Similar to its fate in mammalian cells, OBZ is activated in a phase I reaction resulting in the hydroxylated metabolite 2,4-dihydroxybenzophenone (DHB). Independently, two phase II metabolites were identified as oxybenzone-glucoside (OBZ-Glu) and oxybenzone-(6-*O*-malonyl)-glucoside (OBZ-Mal-Glu) by LC–MS/MS. Formation of these metabolites increased over the experimental period. To our knowledge this is the first time that DHB, OBZ-Glu and OBZ-Mal-Glu are shown to be formed in higher plant tissues. Furthermore, plant defense systems-antioxidative enzymes (SOD, CAT, APOX and POX) were found to be elevated to counteract stress caused by exposure to OBZ. This study presents the huge potential of aquatic plants to cope with benzophenone type UV filters in contaminated water bodies.

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1. Introduction

UV filters have received much attention in recent decades due to their ubiquitous occurrence in surface waters. They are released

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into the environment from skin during bathing and swimming, or indirectly via effluents of municipal waste water treatment plants (Rodríguez et al., 2015). Among the wide range of UV filters, oxybenzone (OBZ) is one of the extensively used sun-blocking agents in many commercially available products (Household Products Database; Li et al., 2007). OBZ has been detected in rivers, lakes, wastewaters and coastal areas at concentrations ranging from ng/L to mg/L (Balmer et al., 2005; Downs et al., 2016; Kameda et al., 2011; Rodríguez et al., 2015). It has been identified as an emerging contaminant due to its persistence in the aquatic environment, accumulation in the biota, and potential threat as endocrine disruptor (Langford et al., 2015; Richardson and Ternes, 2005). For example, bioaccumulation of OBZ has been observed in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), leading to estrogenic, antiestrogenic and antiandrogenic activities (Blüthgen et al., 2012; Coronado et al., 2008). Additionally, OBZ causes the deformation of coral planulae, and increases the rate of coral bleaching (Downs et al., 2016).

Since conventional sewage treatment processes are obviously not adequate for the removal of OBZ, it is necessary to apply alternative approaches to eliminate OBZ and mitigate its threat to aquatic organisms. Previous studies have investigated some removal processes of OBZ, for example, the oxidation of OBZ in water by ferrate (VI) with coexisting constituents or by laccase mediator system (Garcia et al., 2011; Yang and Ying, 2013), ozonation or ultrasound application (Gago-Ferrero et al., 2013; Zúñiga-Benítez et al., 2016). Although these advanced techniques lead to high effluent quality, the cost of installation and operation should be considered carefully before their implementation (Fent et al., 2006; Schröder et al., 2007).

Despite of the aforementioned engineering-based approaches, the “green clean” technique phytoremediation seems promising with respect to high removal rates and degradation capacity of micro-pollutants, easy implementation and lower maintenance, and it does not introduce external chemical substances into the environment (Pilon-Smits, 2005; Schröder et al., 2007). Plants and the organisms in their rhizosphere can be used for phytoremediation in constructed wetlands (CWs) and hydroponic setups (Schröder et al., 2007), and evidence for the role of plants for uptake and metabolism of target compounds is available (Huber et al., 2009; Zhang et al., 2013a; Macherius et al., 2014; Lv et al., 2016). Our previous study reported the uptake and conversion of OBZ by a plant hairy root model system (Chen et al., 2016), however, little work has been done to clarify the capacity of wetland plants to remove and metabolize typical personal care products like sunscreens from water.

This paper focuses on the removal and fate of OBZ by *Cyperus alternifolius* L. growing hydroponically. *C. alternifolius* is a common macrophyte growing best in tropical and subtropical environment (Ebrahimi et al., 2013), where OBZ may occur at high concentration (1.4 mg/L) (Downs et al., 2016). The objectives of this study were to determine the elimination of OBZ from hydroponic solutions, and to assess the translocation of OBZ from roots to shoots of *Cyperus*. Additionally, the potential transformation products of OBZ in different plant parts were to be identified to evaluate the ability of higher aquatic plants to degrade benzophenone type compounds. Furthermore, the tolerance of *C. alternifolius* against OBZ-induced oxidative stress should be explored to characterize the phytotoxicity of OBZ in plants.

2. Materials and methods

2.1. Plant material

Cyperus alternifolius was propagated from seeds purchased from a local provider (Tropica GmbH & Co KG, Münster, Germany). After germination, individual seedlings were transferred to soil in a greenhouse with 12/12 h light/dark cycle at 20/15 °C, and an average humidity of 65%. When the plants were approximately 30 cm tall, the soil was carefully washed off, and the plants were acclimatized hydroponically for four weeks in modified Hoagland nutrient solution, consisting of each 2 mM Mg^{2+}, Ca^{2+}, K^{+}, SO_4^{2-}, and NO_3^{-}, 0.5 mM $H_2PO_4^{-}$, 10 μM H_3BO_3 and Fe^{3+}, 1 μM Mn^{2+}, 0.5 μM Zn^{2+} and Cu^{2+}, 0.1 μM MoO_4^{2-} (Dordio et al., 2009). All the chemicals used were analytical grade.

2.2. Hydroponic exposure system

Plants of uniform size were selected and placed into 250 mL glass vessels containing Hoagland solution spiked with OBZ at concentrations of 5 μM, 25 μM and 50 μM, respectively. Although the higher OBZ concentrations (25 and 50 μM) are not environmentally relevant, they were chosen to induce plant physiological response and to investigate plant tolerance against OBZ, according to similar experiments (Chen et al., 2016; Kotyza et al., 2010). Three replicate vessels were established for each exposure period (i.e., 24, 72 and 120 h) at each concentration, mock plants without OBZ treatment and assays without plants were set up under the same conditions. The entire plant from each replicate was harvested for different treatments at each exposure time, root, stem and leaf samples of each plant were collected separately and frozen at −80 °C for further measurements. The nutrient solutions were collected separately and analyzed to determine the OBZ concentration for each exposure time.

2.3. Analysis of OBZ in growth medium

Nutrient medium samples (1 mL) were collected and filtered through 0.45 μm pore size PVDF syringe filters (Carl Roth, Germany) prior to analysis via HPLC (Varian ProStar 210, Germany). Samples were loaded on an Agilent eclipse XDB-C18 reversed-phase column (5 μm, 4.6 × 150 mm), operated at ambient temperature. Ultrapure water (MilliQ, Millipore Corporation) with 0.1% trifluoroacetic acid was used as mobile phase A and acetonitrile (HPLC grade) with 0.1% trifluoroacetic acid (Carl Roth, Germany) was applied as mobile phase B. OBZ was separated with the following gradient: 0–1 min, 50% A (isocratic), 1–5 min, 90% B (linear increasing), 5–6 min, 50% A (linear decreasing), 6–7 min, 50% A (isocratic). The flow rate was maintained at 1.2 mL/min and OBZ was detected via HPLC with UV detection at 289 nm (Varian ProStar 335, Germany). Removal efficiencies were calculated from declining concentrations in the media over time.

2.4. LC-MS analysis for OBZ and its biodegradation products in plants

The determination of OBZ and its metabolites in plant tissues was carried out using solid phase extraction (SPE) followed by LC-MS analysis. In short, 0.5 g plant material were ground and extracted with 1.5 mL H_2O/acetonitrile (30/70, v/v), the supernatants were then loaded on SPE columns (3 cm^3 60 mg Oasis HLB SPE cartridges, Waters, Germany) according to the procedure of Chen et al. (2016). The LC-MS analysis was conducted on a HPLC system (Varian ProStar 210) coupled to an ion trap mass spectrometer equipped with an electrospray source (Varian 500-MS) as described previously (Huber et al., 2012). Further details are described in Supporting Information Text S1&2.

2.5. Protein extraction and antioxidative enzymes assay

The extraction of soluble protein was carried out according to

the method described by Schröder et al. (2005) including stepwise precipitation with ammonium sulfate. Protein content was determined by the method of Bradford (1976) with bovine serum albumin as a standard protein. All enzyme assays were conducted in a 96-well spectrophotometer (Spectra max Plus 384, Molecular Devices, Germany). The specific activity (except superoxide dismutase) is expressed as the enzymatic formation or consumption of one mole product per second [katal] in the enzyme extracts.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as the change from epinephrine to adrenochrome at 480 nm for 5 min (10% enzyme concentration per reaction). One unit of SOD activity is defined as the amount of protein required for 50% inhibition of adrenochrome formation (Polle et al., 1989).

Catalase (CAT, EC 1.11.1.6) activity measurement was based on the decrease in absorption at 240 nm (ε 0.036 $mM^{-1}cm^{-1}$) due to the consumption of H_2O_2 (6.7% enzyme concentration per reaction) (Verma and Dubey, 2003).

Peroxidase (POX, EC 1.11.1) activity was assayed at 420 nm (ε 26.6 $mM^{-1}cm^{-1}$) using guajacol (25.05 mM per reaction) as a substrate (5% enzyme concentration per reaction) (Diekman et al., 2004).

Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was measured according to the method described by Lyubenova et al. with modifications (2015). Each reaction contained 1 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and enzyme extract in 50 mM KH_2PO_4/K_2HPO_4 buffer (10% enzyme concentration per reaction), and the activity was determined at 290 nm (ε 2.8 $mM^{-1}cm^{-1}$).

Glutathione reductase (GR, EC 1.6.4.2) activity was evaluated by the extinction at 340 nm (ε 6.2 $mM^{-1}cm^{-1}$) resulting from the consumption of NADPH (5% enzyme concentration per reaction) (Vanacker et al., 1998).

2.6. Data analysis

Statistical analyses were performed with the software GraphPad Prism v5.0. Two-way analysis of variance (ANOVA) with Bonferroni post-test was applied to determine significant differences between groups. Comparisons among concentrations of OBZ and its metabolites were considered significantly different for $p < 0.05$, and data shown in section 3.4, which depicted the changes in enzyme activities (%) between OBZ treated and untreated plant were considered significant at $p < 0.01$.

3. Results and discussion

3.1. Removal of OBZ from hydroponic medium

Hydroponic experiments were performed to evaluate the ability of *C. alternifolius* to remove OBZ. The initial concentrations were established based on the maximum level detected in water bodies (1.4 mg/L) (Downs et al., 2016). Generally, removal of xenobiotics in hydroponic systems has been be attributed to abiotic (photodegradation, evaporation, hydrolysis) and biotic processes (plant uptake and microbial degradation) (Yan et al., 2016a; Zhang et al., 2016). For OBZ, photodegradation and volatilization were not expected to occur due to the photostability of OBZ designed for blocking UV radiation (Liu et al., 2011) and low volatility of this compound. In control vessels without plants (same nutrient medium as other treatments), OBZ concentrations remained constant (Fig. S1) over the incubation time indicating that the adsorption on vessel walls was also negligible. It may be concluded that biotic processes accounted for the main removal process.

The OBZ concentration in the medium dropped dramatically toward the end of the incubation time, and more than 73.9 ± 9.1% of OBZ were removed from media for all three different initial concentrations. A rapid decrease of OBZ concentration was observed within 24 h for the 5 μM treatment yielding an initial removal efficiency of 49.5 ± 6.4%. OBZ was then eliminated continuously from medium as shown in Fig. 1. Ferreira et al. (2016) have demonstrated that the presence of *Spartina maritima* (Curtis) Fernald stimulates the remediation of OBZ at a removal rate of 62%. Recently, high removal efficiency of OBZ up to 97% has been reported in a constructed wetland applied as secondary treatment system (Matamoros et al., 2007). In the study of removal of four pharmaceutically active compounds by *C. alternifolius*, Yan et al. (2016a) observed higher removal efficiencies as the initial concentrations decreased. Similar to their observations, the present results showed that OBZ was eliminated more efficiently at lower initial concentration. The treatment spiked with 5 μM OBZ showed the highest removal efficiency of 86.5%, followed by the efficiency of 81.4% at 25 μM OBZ treatment.

3.2. OBZ uptake by root and translocation to shoot

To study the fate of OBZ within plants, the compound was quantified in root, stem and leaf tissues of *C. alternifolius* exposed to OBZ spiked solutions. OBZ could be detected in all tissues for all concentrations, and root samples from plants treated with 50 μM OBZ showed the highest OBZ concentration (Fig. 2). This result fits to a recent carbamazepine removal study where higher accumulation occurs in roots exposed to higher initial concentrations (Dordio et al., 2011; Zhang et al., 2013b).

Many organic xenobiotics move into plant tissues by diffusion which mainly depends on their hydrophobicity (Pilon-Smits, 2005). OBZ is a moderately hydrophobic compound with a log K_{ow} of 3.52, and was taken up continuously by the roots of *C. alternifolius*. This is consistent with the general agreement that compounds with log K_{ow} between the range of 1–3.5 are lipophilic enough to be taken up efficiently (Dietz and Schnoor, 2001). Particularly, OBZ was accumulated up to 58.4 ± 8.6% for 5 μM treatment after 120 h (Fig. 2). This finding fits to previous studies that plant uptake occurs rapidly and within a rather short time (Bartha et al., 2010; Zhang et al., 2013c). A previous review mentioned that most of the studied pharmaceutical and personal care products accumulate in or on roots of plants (Miller et al., 2016). It has been demonstrated that the remediation of OBZ was enhanced by adsorption at plant root surfaces (Ferreira et al., 2016). However, the amount of OBZ detected in plants during this study was less than that removed

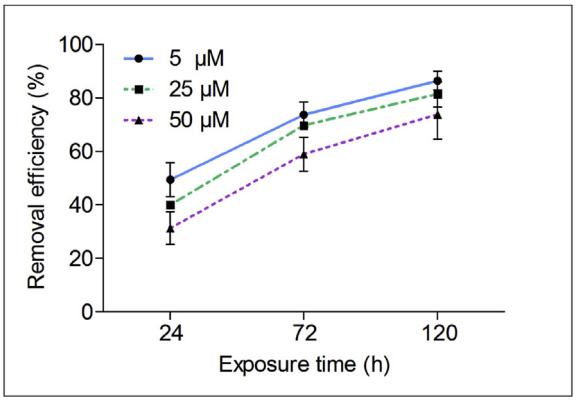

Fig. 1. Removal of oxybenzone (OBZ, 5, 25 and 50 μM) from hydroponic culture medium by *C. alternifolius* at each exposure time. Removal efficiency (%) = (Ci−Ct)/Ci × 100%, where Ci is the initial OBZ concentration in the medium (mg L^{-1}), Ct is the OBZ concentration (mg L^{-1}) detected in the medium at exposure time t. Error bars indicate standard deviation (n = 3).

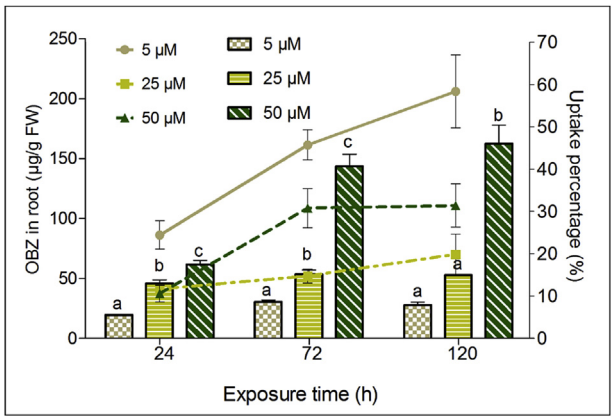

Fig. 2. Oxybenzone (OBZ) concentration in roots (column) and OBZ uptake percentage (line), the uptake percentage of OBZ (%) = the mass of OBZ taken up by roots of exposed *C. alternifolius* (μg)/the initial mass of OBZ in the hydroponic medium (μg), the mass of OBZ in roots = roots weight (g) × OBZ concentration in root (μg g^{-1} fresh weight (FW)) Error bars indicate SD (n = 3).

from nutrient media. This should mainly result from transformation to metabolites.

OBZ is assumed to be taken up and translocated to the aerial part of plants. After 120 h treatment, OBZ was detected in stems at concentrations ranging from 1.9 ± 0.2 to 34.2 ± 2.6 μg/g (FW) (Fig. 3). Accumulation in stems which have the highest biomass, accounted for 9.6 ± 1.6 to 51.5 ± 3.8% of the total OBZ detected in the plant. A recent study of phytodegradation of ethanolamines revealed that *C. alternifolius* has the highest efficiency, and could completely remove monoethanolamine after 120 h with an initial concentration of 18 mM, and accumulation of ethanolamines was found mainly in stems (Dolphen and Thiravetyan, 2015). OBZ concentrations in leaves were much lower compared to those in roots, which corresponds to the observation of Lv et al. (2016), who found a higher accumulation in roots of *Typha latifolia* L. for the pesticides imazalil and tebuconazole with log K_{ow} similar or higher to OBZ. Based on the findings of Briggs et al. (1982) that maximum translocation to shoots occurred for compounds with log K_{ow} centered around 1.8, OBZ might be too hydrophobic for efficient translocation. However due to longer incubation, OBZ in leaves increased over time and finally reached 0.61 ± 0.03–4.4 ± 0.5 μg/g (FW) (Fig. 3). Significant difference ($p < 0.05$) for shoot bioaccumulation was observed among three initial concentrations.

3.3. Metabolites

OBZ can be effectively metabolized in plant cells. To demonstrate OBZ metabolism in *C. alternifolius* tissues, LC-MS^2 was conducted for metabolite identification. Fig. 4 presents the chromatogram of OBZ and its metabolites which were more polar and therefore eluted earlier than OBZ (spectra of OBZ and metabolites are presented in Fig. S2). The first metabolite peak was identified as oxybenzone-glucoside (OBZ-Glu) with its pseudomolecular ion at *m/z* 413 $[M+Na]^+$, the second peak was detected at *m/z* 499 $[M+Na]^+$ which corresponds to oxybenzone-(6-*O*-malonyl)-glucoside (OBZ-Mal-Glu). These two phase II metabolites have also been detected in our previous study on the OBZ pathway in a horseradish hairy root culture (Chen et al., 2016). However, a third metabolite peak was obtained during the MS^2 collision experiment (inserted graph in Fig. 4), which eluted at 7.7 min with a mass transition of $[M+H]^+$ *m/z* 215-137. This peak was confirmed as phase I product 2,4-dihydroxybenzophenone (DHB) due to the same retention time and fragmentation pattern generated from a standard DHB solution. DHB is formed via dealkylation of the methoxy-side chain at the para-position of OBZ, generally mediated by cytochrome P450 monooxygenase enzymes (Okereke et al., 1994). This phase I activation is in consistent with that found for OBZ destruction in mammalian liver, but it has been reported that OBZ undergoes more extensive hydroxylation to form 2,2-dihydroxy-4-methoxybenzophenone (DMB) and 2,3,4-trihydroxybenzophenone (THB), and conjugation with glucuronide or sulfate by phase II enzymes (Wang and Kannan, 2013). However, phase I activating reactions do not always result in products with decreased toxicity, and DHB is suggested to have more estrogenic activity than OBZ (Fediuk et al., 2012; Nakamura et al., 2015). On the other hand, conjugation with reactive molecules in phase II metabolism is supposed to increase the molecular mass, improve hydrophilicity and modify the parent compound into non-toxic or less toxic products (Coleman et al., 1997; Zamek-Gliszczynski et al., 2006).

DHB concentration was quantified in each tissue using a standard curve. As shown in Fig. 5a, formation of DHB increased significantly in roots after exposure at 50 μM for 72 h, and DHB concentration in leaf was significantly higher (11.2–39.3-fold) than the other two concentrations over the exposure time. Formation of OBZ-Glu and OBZ-Mal-Glu proceeded without activation by phase I reactions, probably because OBZ already has a functional hydroxyl group suitable for phase II transformation. Interestingly, these products occurred not only in roots but also in shoots, and the

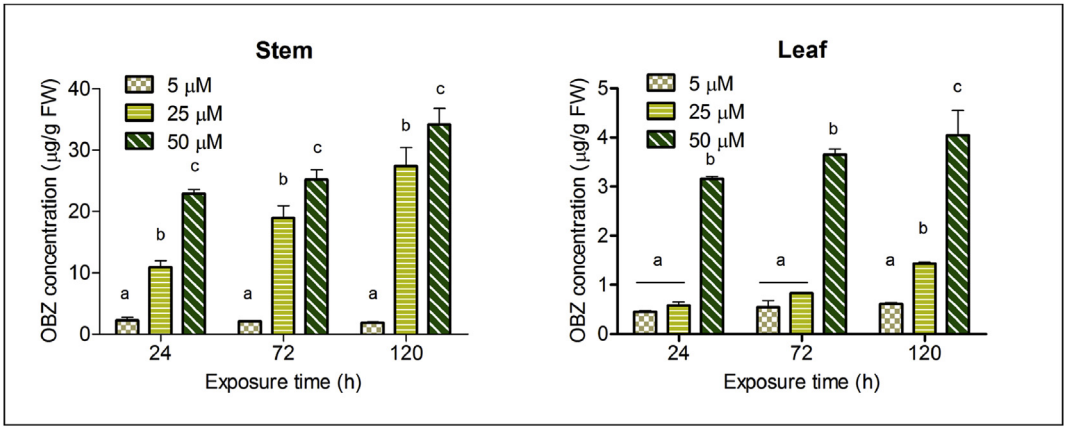

Fig. 3. Concentrations of oxybenzone (OBZ) detected in stems and leaves of *C. alternifolius*. Lowercase letters indicate significant differences among three different concentrations according to ANOVA at $p < 0.05$. Error bars indicate SD (n = 3).

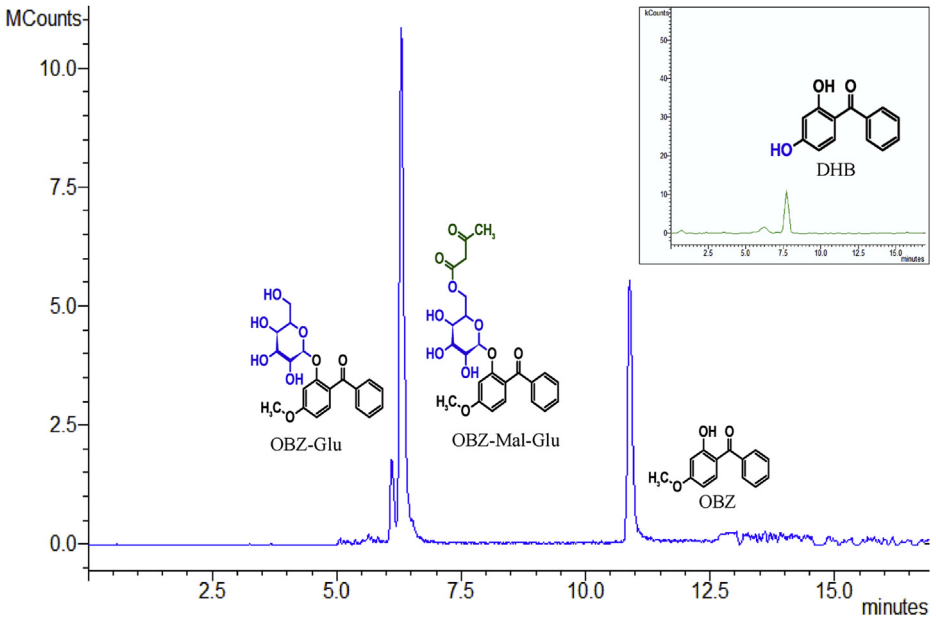

Fig. 4. LC-MS/MS chromatogram of samples containing oxybenzone (OBZ), oxybenzone-glucoside (OBZ-Glu) and oxybenzone-(6-O-malonyl)-glucoside (OBZ-Mal-Glu), obtained at selected product ion *m*/*z* 151(*SIM* mode). Inserted graph presents LC-MS/MS chromatogram of 2,4-dihydroxybenzophenone (DHB) (*SIM* mode at *m*/*z* 137). The analysis was done in positive ionization mode. The scheme below depicts the chemical structure of OBZ and proposed structure for the metabolites detected in *C. alternifolius*.

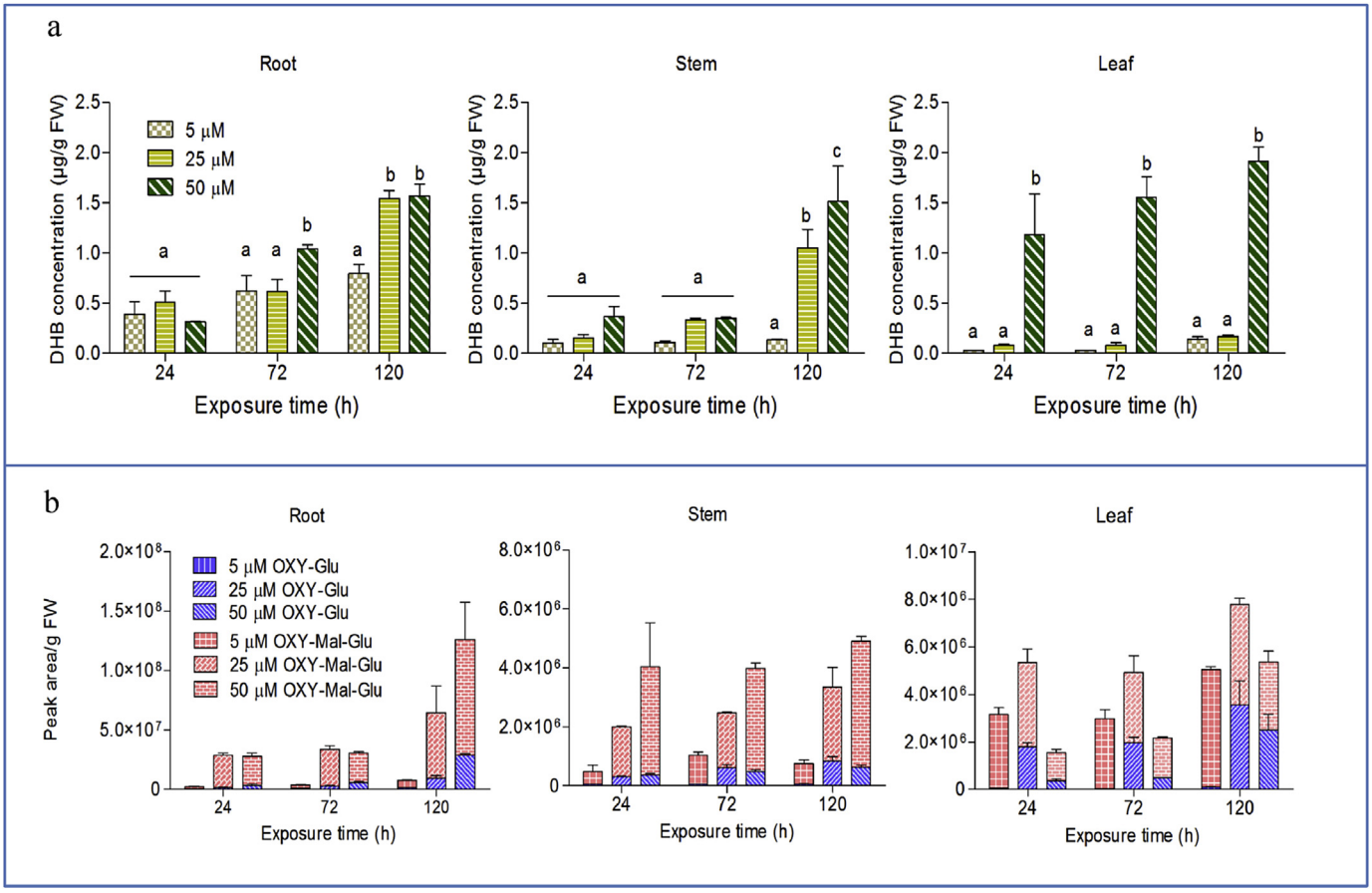

Fig. 5. Formation of oxybenzone metabolites: 2,4-dihydroxybenzophenone (DHB) (a), oxybenzone-glucoside (OBZ-Glu) & oxybenzone-(6-O-malonyl)-glucoside (OBZ-Mal-Glu) (b) in roots, stems and leaves of *C. alternifolius* after 24, 72 and 120 h incubation. Values are mean of three parallel individuals, error bars indicate SD. Lowercase letters indicate significant differences among three different concentrations according to ANOVA at $p < 0.05$.

amount of OBZ-Glu and OBZ-Mal-Glu was higher in roots than in shoots. Still, the amount increased with exposure time in all treated assays. Due to the lack of reference materials of OBZ-Glu and OBZ-Mal-Glu, their concentrations are given in peak area as arbitrary units in Fig. 5b, with OBZ-Mal-Glu accounting for a major part. Malonyl glucosides (Mal-Glu) are characterized as stable and more hydrophilic, and malonylation is one of the key reactions to promote efficient sequestration of metabolites in the vacuole, moreover, the expression of malonyltransferase (enzyme responsible for malonylation) gene is induced by oxidative stress (Taguchi et al., 2010). Hence it seems likely that plants under moderate oxidative stress would increase this branch of a sequestration pathway.

3.4. Effects of OBZ on C. alternifolius antioxidative systems

Abiotic stress caused by the plant exposure to xenobiotics may disrupt the steady-state level of reactive oxygen species (ROS) in plants. Over-production of ROS may cause damages on plant lipids and proteins (Halliwell and Gutteridge, 1984), and may affect plant detoxification of xenobiotics. Antioxidants and enzymes are engaged to scavenge excessive ROS, among them major enzymes including SOD, APOX and CAT considered as first line of defense

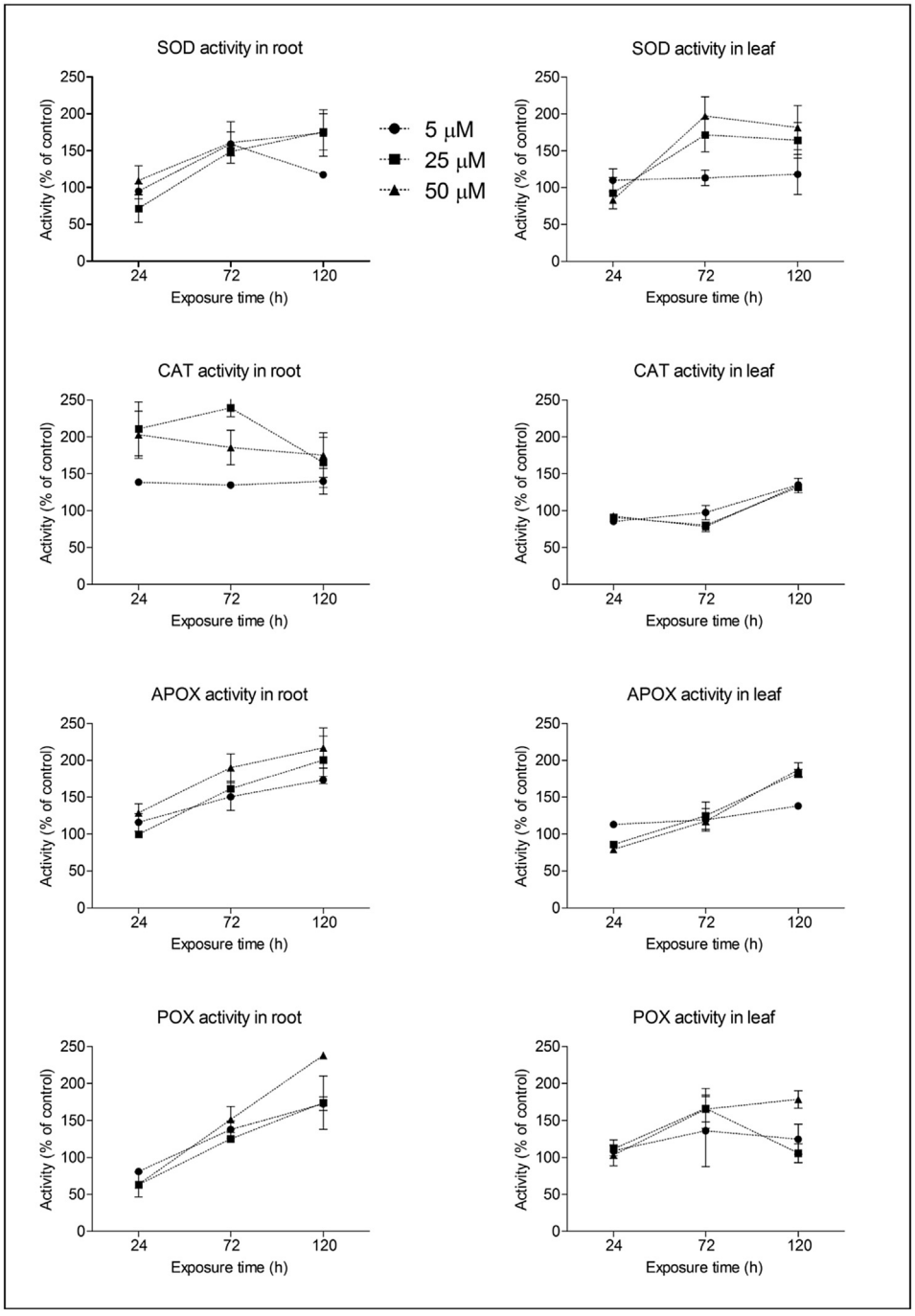

Fig. 6. Distribution of antioxidative enzyme activities in tissues of *C. alternifolius* exposed to different concentrations of oxybenzone (OBZ) at each sampling time. Enzyme activities are depicted relative to controls without OBZ treatment. Data are means of three replicates. Error bars indicate SD (n = 3).

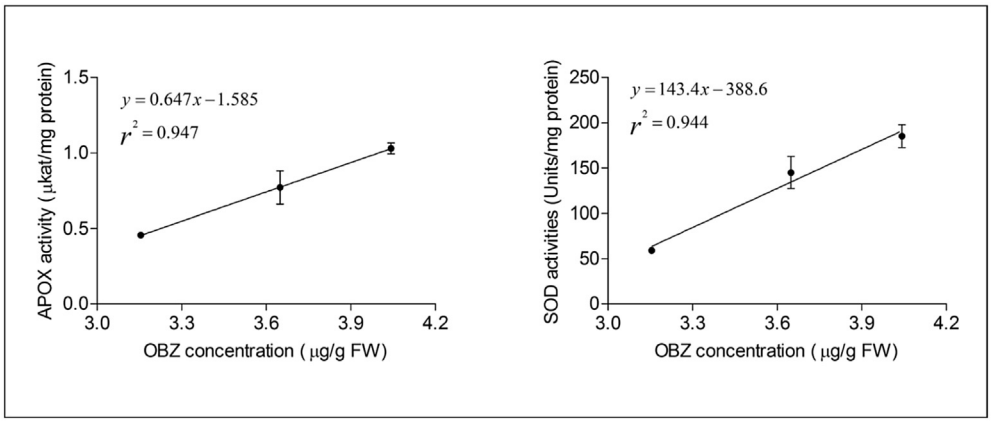

Fig. 7. Example of correlation between activities of ascorbate peroxidase (APOX) and superoxide dismutase (SOD), two antioxidative enzymes, and oxybenzone (OBZ) concentration in the plant tissues (at three sampling time 24 h, 72 h and 120 h from left to right). Activity of SOD and APOX correlates linearly to the amount of OBZ accumulated in leaves (at 50 μM OBZ treatment). Values are mean of three replicates.

against oxidative stress (Mittler, 2002). The balance of these enzyme activities is important in regulating the stable status of O_2^- and H_2O_2, and the activity levels are considered as indicators of stress levels that plants are subjected to (Mittler, 2002). In our study, after the uptake of OBZ in plants, activities of the aforementioned enzyme activities were determined in root and leaf tissues to characterize the effects of OBZ on *C. alternifolius*. The results are summarized in Fig. 6.

SOD is expressed in many cell compartments and converts O_2^- into H_2O_2 to maintain O_2^- level in cells low (Mittler, 2002). Its activity in roots was enhanced after 72 h treatment compared to control. In the following hours, the activity returned to control level in the 5 μM exposure, indicating the counteraction of an oxidative burst. Whereas at 25 and 50 μM exposure concentrations, SOD activity remained elevated to 176% and 174% of control after 120 h, which could be explained by the increase of O_2^- within cells by continuous accumulation of OBZ in roots. In leaves, maximum increase in SOD activities was observed for 50 μM after 72 h. The activities in leaves were much lower compared to roots, probably because roots are the primary organs exposed to OBZ, and higher accumulation of OBZ in roots led to higher stress. Schröder et al. (2005) have reported similar findings that rhizomes have ten-fold higher activities than that in leaves of *Phragmites australis* Trin.

CAT is expressed in peroxisomes and is crucial for degrading H_2O_2. Its activity in roots exposed to OBZ was enhanced strongly to 239% after treatment with 25 μM OBZ, and longer incubation resulted in decline of activities. OBZ application did not cause significant changes of CAT activities in leaves. APOX involved in the ascorbate-glutathione cycle is found in almost all cellular compartments and H_2O_2 is reduced to water by APOX using ascorbic acid as electron donor (Mittler, 2002). APOX activities in general were elevated in roots and leaves with a maximum increase of 216% and 187% at the end of the incubation period.

POX also contributes to removal of ROS by using phenolic compounds as electron donors to reduce H_2O_2. In roots, its activity was depressed to 63% upon treatment followed by continuous increase to 238% of control level. POX activity in leaves was not influenced at 5 μM OBZ treatment, but was enhanced at higher concentrations. A rapid decrease of POX activities to initial levels occurred in incubations with 25 μM OBZ in leaves harvested after 120 h treatment, whereas 50 μM OBZ still triggered an increase to 179%, indicating that this treatment might induce higher amounts of H_2O_2 which need to be buffered. Previous studies reported POX induction caused by xenobiotics in leaves of several plant species. Bartha et al. (2010) found a strong elevation of POX activity up to six fold in leaves of *Brassica juncea* L. after 168 h exposure to acetaminophen. A 218.2% increase of POX activity was observed in *C. alternifolius* grown in simulated CWs under the influence of pharmaceuticals (Yan et al., 2016a). Besides cleavage of H_2O_2, POX is also able to oxidize xenobiotics. Huber et al. (2016) described the activation of diclofenac by POX during the detoxification process. Thus, also in the case of OBZ, the development of an oxidative burst and involvement in transformation of OBZ to DHB might induce an increased activity of POX.

The present observations showed that antioxidative enzymes were elevated as a defense against the oxidative burst induced by the presence of OBZ. A strong link between OBZ accumulation in tissues and induction of defense enzyme activities was observed. Fig. 7 as an example shows that activities of APOX and SOD had positive linear correlations (R^2 0.947 and 0.944, respectively) to the amount of OBZ accumulated in leaves. Recent proteomics approaches on the effects of four pharmaceuticals on *C. alternifolius* in CWs suggested the remarkable tolerance of this plant species, showing that upregulation of proteins such as Cu/Zn SOD involved in antioxidative defense are effective to enhance stress tolerance (Yan et al., 2016b). Additionally, based on the results of Mittler et al. (2001) that C4 plants show better performance under stress, the physiological adaptations of *C. alternifolius* as a C4 plant species might also favor the protection against stress.

4. Conclusions

The present study proved that the presence of plants will improve the removal of OBZ from water and provided insights in the fate of OBZ in higher plants. Our findings show fast and efficient depletion of OBZ from media, and hydroponic systems in the absence of soil demonstrated the role of plant during uptake and metabolism. OBZ concentrations in the plants were found to be ranked as roots > stems > leaves. Hence, OBZ has the potential to be translocated to the upper part of plant; therefore, a high removal flux of OBZ is expected due to the season dependent occurrence of OBZ and perennial growth of *C. alternifolius*. Despite of the phase I hydroxylated product, the two independently formed phase II metabolites are considered to be more water soluble and less toxic, and favorable for OBZ sequestration in the plant. Distribution of enzyme activities indicates that exposure to OBZ caused mild stress to *C. alternifolius*. 5 μM OBZ, which is equivalent to the maximum amount of OBZ detected in environment, generally resulted in less stress compared to the other higher concentrations. The induced enzyme activities indicate their role in defense and adaptation

against stress resulting from prolonged OBZ exposure.

The current study shows that *C. alternifolius* is an appropriate candidate macrophyte for the removal of OBZ from CWs. The uptake and accumulation of OBZ as well as its transformation to conjugates with lower toxicity in *C. alternifolius* provide evidence for the plant's possible application in phytoremediation. As recommended in all cases of phytoremediation, harvested biomass should be collected and might be used for bioenergy purposes to avoid leaching of unmetabolized OBZ from decaying plant material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.05.072.

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The following is the supplementary data related to this article:

Text S1 LC-MS analysis for OBZ and its biodegradation products in plant

The separation was achieved with a Phenomenex HYDRO-RP column (C18, polar endcapped; particle size 4 μm; 50 mm × 2.0 mm) using H_2O acidified with 0.1% formic acid as buffer A and acetonitrile acidified with 0.1% formic acid as buffer B at a flow rate of 0.3 mL/min, using the following elution gradient: isocratic at 97% of A (0-2min), 2–10 min linear gradient from 3% to 95% of B (2-10min), isocratic at 95% of B (10-12min), linear gradient from 5% to 97% of A (12–12.5 min) and isocratic at 95% of A (12.5–17 min). The MS was operated in the positive ion mode with conditions adjusted to: capillary voltage, 63 V; needle voltage, 4500 V; drying gas temperature, 300 °C. MS/MS fragmentation was performed by collision-induced dissociation using helium as the collision gas.

Text S2 Solid phase extraction (SPE) optimization--extraction recovery

To find the best SPE conditions which yield high extraction recoveries, different extraction solvents, cartridges and elution conditions were tested. For this purpose, root extracts free from OBZ were spiked with extraction solvents (d.t. water and acetonitrile, v/v at 50/50, 30/70 and 20/80, respectively) containing 50 μM OBZ. The samples were then treated in the ultrasonicator for 5 min and centrifuged at 13,000 ×*g* and 4°C for 30 min. Supernatants were collected and filtrated through 0.45 μm pore size PVDF syringe filters (Carl Roth, Germany) prior to loading on them on solid phase extraction (SPE) columns (3 cm^3 60 mg Oasis HLB SPE cartridges, Waters, Germany) for further purification. Cartridges had been conditioned with 3 mL of methanol and equilibrated with 3 mL of water (MilliQ) prior to use. 0.5 mL of samples were passed through the cartridges and 3 mL water (MilliQ) were used to flush impurities. The cartridges were then dried under vacuum for 10 min, and analytes were subsequently eluted with two 0.7 mL and one 0.6 mL aliquots of acetonitrile.

The absolute extraction recoveries of OBZ were calculated as percentage ratios of peak areas of the eluted compounds from root extracts, to the respective peak areas of the compounds in a non-extracted standard solution. The standard solution contained the same concentrations of OBZ as the extracted samples, analysis were done by HPLC. The recoveries ranged from 70% to 95%, among which water to acetonitrile v/v 30/70 presented best recovery. 3 cm3 60 mg Oasis HLB SPE cartridges were selected due to their high recoveries for both OBZ and its metabolites.

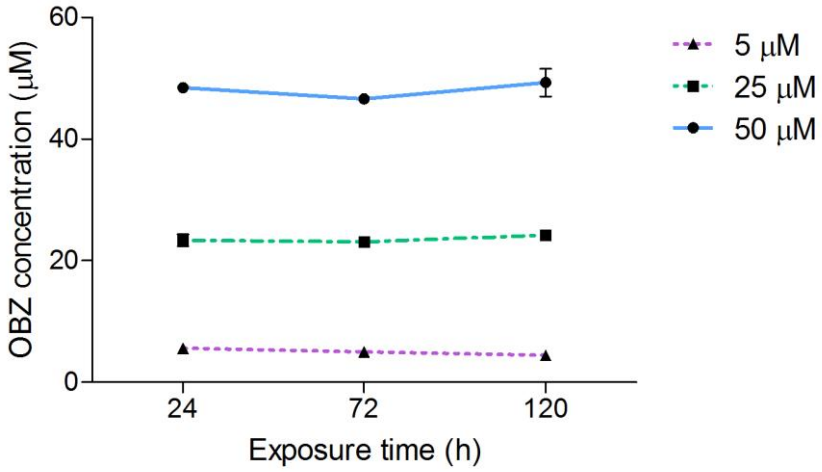

Figure S1 OBZ concentration (μM) in medium without plants over incubation time. No statistical difference among exposure time for each concentration according to ANOVA at $p < 0.05$. Error bars indicate standard deviation (n=3).

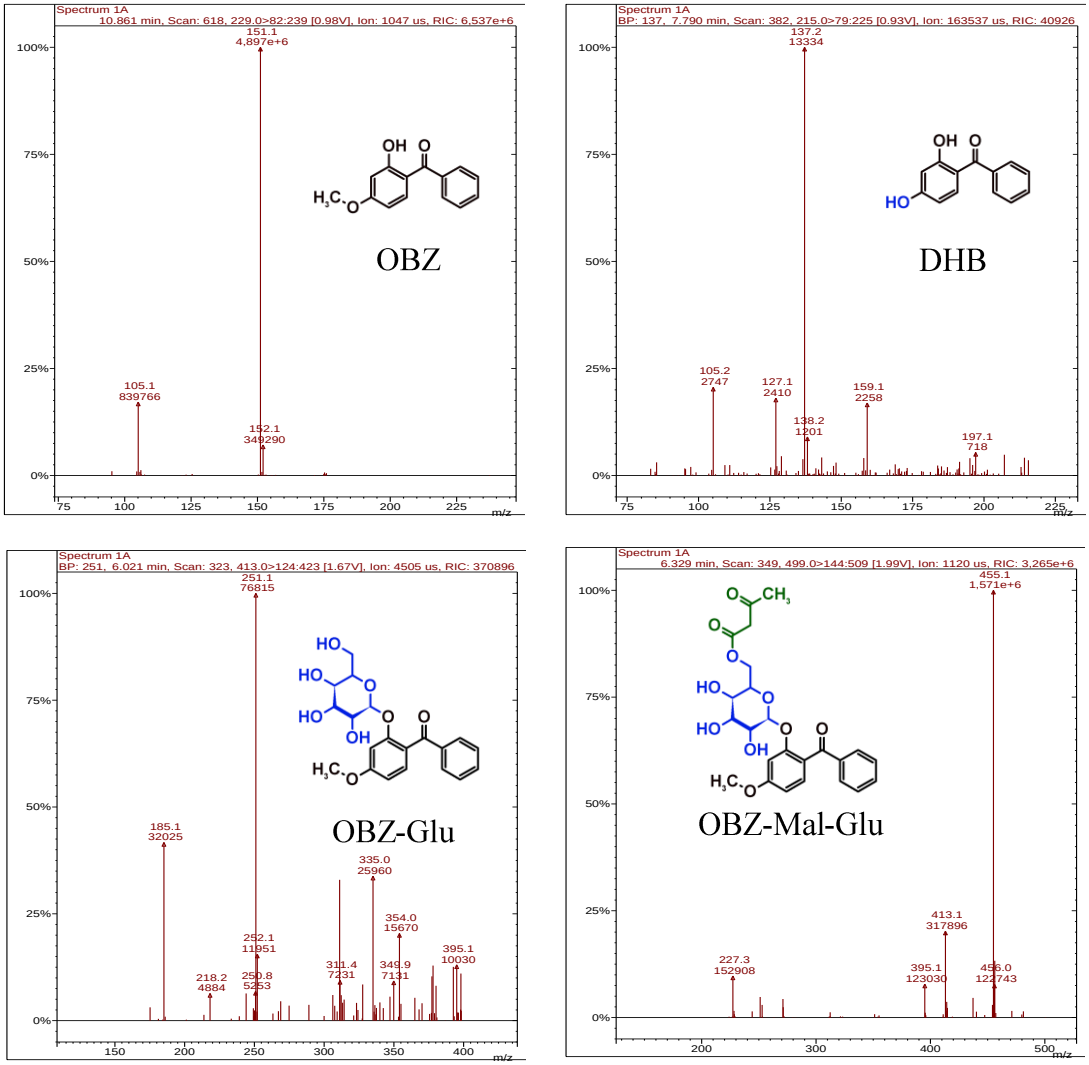

Figure S2 MS/MS spectra of OBZ (m/z 229), DHB (m/z 215), OBZ-Glu (m/z 413) and OBZ-Mal-Glu (m/z 499).

Uptake and transformation of Oxybenzone in the presence of TiO_2: Impact of nanoparticles on the plant remediation of organic UV filter

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Abstract

Oxybenzone (OBZ) present in the environment as an emerging contaminant may occur jointly with the nanoparticle TiO_2 due to the typical composition of sunscreens. Thus, the effects caused by TiO_2 must be considered when investigating the environmental fate of sunscreens and also when plant performance on remediation of OBZ is scrutinized. Toxicity effect of OBZ and TiO_2 on plant development was evaluated by germination rate and root length of tomato and barley. Results showed that OBZ significantly inhibited germination rate of tomato seeds, while no effect was observed for germination of barley seeds. Interestingly, co-exposure with TiO_2 lowered the toxicity of OBZ on the tomato seedlings as there were no differences on germination rate and root length between co-exposure and control treatments. Moreover, growth inhibition test with *Lemna minor* showed that addition of TiO_2 even enhanced plant growth by increasing the frond area. Furthermore, influence of TiO_2 (3 mg/L) on removal of OBZ (5 µM) by plants was examined with respect to the variations in uptake and metabolism of OBZ in a hairy root culture system. Co-exposure to TiO_2 amplified the accumulation of OBZ in plants; while transformation to OBZ metabolites was less efficient when TiO_2 had been added. Therefore, it can be concluded that Ti-nanoparticles may generally reduce the phyto-toxicity of OBZ and increase the uptake of this compound in phytoremediation, while the interaction on the transformation capacity should be considered when applying phytoremediation for UV-filter contaminated water.

Keywords: oxybenzone, TiO_2, uptake, transformation, hairy roots

1 Introduction

UV filters are common components among the personal care products (PCPs), and they have been suspected to become emerging contaminants due to their massive release during recreational activities in fresh and seawater as well as from effluents of wastewater treatment plants fed with municipal waste water [1]. Commercial sunscreens may contain chemical (organic, absorb UV radiation) and physical (inorganic, reflect UV) filters [2]. Oxybenzone (OBZ) is a typical organic ingredient added in the sun protective products, over 81% of the 231 PCPs collected from United States and China contain this compound [3]. The widespread use of OBZ has led to its release into the environment and today it is one of the most frequently detected UV filters in surface water and wastewater [1,4–7]. Worldwide, highest concentrations up to 1.395 mg/L of OBZ were detected along Trunk Bay in Virgin islands [8]. Accumulation of OBZ has also been reported in organisms involved in the aquatic food chain [1,7] and the compound has been proved *in vivo* as an endocrine-active agent to fish [9]. Moreover, the presence of OBZ has been associated with the deterioration of coral reefs [10]. Titanium dioxide (TiO_2), a well-known nanomaterial, is widely incorporated as an inorganic UV filter in cosmetics and industrial products, which is also applied extensively to photo catalyze breakdown of environmental pollutants [2]. The US annual production of nano-TiO_2 is estimated to reach 2.5 million metric tons in 2025 [11]. Both, the growth of production and application inevitably enhance the entry of TiO_2 into the environment. Recently 3000 µg/L of titanium has been detected in raw sewage water [12], which ultimately could lead to interactions with the environment and living organisms.

Phytoremediation has been recommended as a suitable technology to cope with emerging contaminants in many compartments of the aquatic environment. Previous studies focused primarily on the single treatment of OBZ by aquatic higher plants [13], however since commercial sun protective products normally contain several components, including nanoparticles, in this study, OBZ and TiO_2 were chosen as the compounds of interest which are often combined to provide strong photo protection.

Nanoparticles have been demonstrated to be beneficial for the delivery of biomolecules into plant cells [14], and at the same time they have been reported to alter the bioavailability and fate of other contaminants. The accumulation of the pesticide chlordane in crops increased 34.9% when exposed to C_{60} fullerenes [15]. Zhang et al. demonstrated the enhanced bioaccumulation of cadmium in carp in the presence of TiO_2 [16]. Also, the

nutrient uptake patterns in *E. canadensis* were altered with the addition of TiO_2, and concentrations of all the elements showed significant correlations with each other [17].

Considering the influence of nanoparticles on many organic chemicals, it is essential to investigate the treatment efficiency of OBZ by plant with respect to the nano-TiO_2. To highlight the above aspects, the toxicities of OBZ and TiO_2 on plants were estimated by evaluating germination rate and root length of barley and tomato seedlings. In addition, growth inhibition was tested with *Lemna minor* growing hydroponically in microplates, and the resulting impact on frond area and photosynthetic pigments was determined. More importantly, a well-established horseradish (*Armoracia rusticana*) hairy root system (HRs) was selected for the current study, with the advantages of fast growth, free of bacterial interference. Since the results obtained from HRs have been proven to be reliable and can be extended to intact plants, this system represents an appropriate approach for characterizing the fundamental processes in plants. The influence of TiO_2 on uptake and transformation of OBZ was therefore studied in HRs with and without addition of TiO_2 in the aqueous suspensions. Furthermore, enzyme activities were measured to evaluate the effects of both compounds on the performance of the plant detoxification system.

2 Materials and methods

2.1 Seed germination tests

Seeds of barley (*Hordeum vulgare*) and tomato (*Lycopersicon esculentum*) were placed on wet filter paper and germinated in suspensions containing either 5 µM OBZ or 3 mg/L TiO_2 alone, or 5 µM OBZ mixed with 3 mg/L TiO_2, respectively. Seeds free of both compounds were regarded as control. TiO_2 was suspended in ultrapure water (MilliQ, Millipore Corporation), the suspension was sonicated twice for 2 min, using a ultrasonic homogenizer (SonoPlus HD 2070, Bandelin, Germany) at an energy of 40 Watt [18], the suspension was subsequently filtered through membrane filter of 220 nm (PVDF, Carl Roth GmbH, Germany) to eliminate large agglomerates [19]. Two plates were prepared for each treatment; in every plate 15 seeds with same shape were arranged with same space. All seeds were incubated at room temperature, barley seeds were placed in dark for 3 days and tomato seeds were exposed with a 15/9 h light/dark cycle for 10 days. The number of germinated seeds was counted and the root length was recorded by a image processing software (ImageJ).

2.2 Microbiotest with *Lemna minor*

A microbiotest was designed according to the commercial protocol with modifications [20]. In short, *Lemna minor* plantlets with homogeneous frond size were selected and transferred to 96-well microplate containing Steinberg medium [21]. Each well contained one frond, 24 wells were integrated for each treatment. Treatments including OBZ/TiO_2 alone or in mixture were set up similar to those in the seed germination tests. Media in control wells were without xenobiotic pollution. The plate was incubated for 3 days at 25 °C. Digital photos were taken at both beginning and end of the incubation, and the frond area at these two time points was measured with the help of ImageJ. The growth of *Lemna* fronds was evaluated by the relative growth rate, which is calculated on the basis of changes in frond area determined during the course of the 3-day exposure period. Additionally, chlorophyll and carotenoid contents of corresponding fronds after 7 d incubation were determined as described previously [22], briefly, 0.05 g freshly ground frond powder was immersed with 0.6 mL cold 95% ethanol, after 1 h storage in a dark fridge (4 °C) the samples were centrifuged at 4000 g for 1 min, the resulting supernatant was collected while the pellet was dissolved in 95% ethanol, stored and centrifuged again, the same procedure was repeated twice until the fronds were virtually pale. Supernatants from each centrifugation were collected and combined; the samples were measured spectrophotometrically at 664.1, 648.1 and 470 nm according to the method of Lichtenthaler and Buschman [23]. The pigment contents were expressed as µg/g fresh weight.

2.3 Hairy root culture experiment

Hairy root culture of horseradish had been obtained formerly by transformation of nodal segments by *Agrobacterium rhizogenes* strain A4 [24]. After sub-division, roots obtained from the same generation were adopted and grown in 250 mL Erlenmeyer flask with 100 mL Murashige and Skoog medium with addition of sucrose inositol and thiamine for 10 days. The experiment was initiated by incubating the roots in medium containing 5 µM OBZ supplemented in the presence or absence of 3 mg/L TiO_2, roots added independently with 3 mg/L TiO_2, control roots free of both compounds and medium without roots were set up under the same conditions. Triplicate samples of roots and medium from each treatments (including control) were harvested at 0, 2, 4 and 7 days, respectively. Root samples were frozen in liquid nitrogen and stored at -80 °C. All the chemicals used were analytical grade.

2.4 Extraction of roots

OBZ and its metabolites were extracted according to the method described previously [25]. In short, 0.5 g of plant material were ground and extracted with 1.5 mL H_2O/acetonitrile (30/70, v/v). After 5 min ultrasonication and 30 min centrifugation at 13,000 ×g. Supernatants were purified with 3 cm^3 60 mg Oasis HLB solid phase extraction (SPE) columns. Growth medium was filtered with PVDF syringe filters prior to analysis.

O-glucosyltransferase (*O*-GT, E.C. 2.4.1.x) was extracted based on the method described previously [26]. Three grams of hairy roots were homogenized and extracted with 100 mM sodium phosphate-buffer pH 6.5 containing 10 mM DTE, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 1% PVP K90 at 4°C for 30 min. After centrifugation at 15,000 ×g for 30 min at 4°C, proteins in the supernatant were precipitated progressively by addition of ammonium sulphate to 40% and 75% saturation and centrifuged at 18,500 ×g for 30 min at 4°C, respectively. Consequently, the pellets were resuspended in 2.5 mL 200 mM Tris/HCl buffer with 2 mM $MgCl_2$ and 1 mM DTE, pH 7.3. Proteins were desalted by size exclusion chromatography through PD 10 columns (GE Healthcare, UK) and stored at -80°C before use.

2.5 LC-MS analysis

OBZ and metabolites were determined with a HPLC system (Varian ProStar 210) coupled to an ion trap mass spectrometer (Varian 500-MS). A Phenomenex HYDRO-RP column (C18, polar endcapped; particle size 4 µm; 50 mm × 2.0 mm) was applied for separation of analytes using H_2O with 0.1% formic acid as mobile phase A, acetonitrile with 0.1% formic acid as mobile phase B with following gradient: 0-2 min 97% Buffer A; 2-10 min 95% Buffer B; 10-12 min 95% Buffer B; 12-12.5 min 97% Buffer A; 12.5-17 min 97% A. The flow rate was 0.3 mL/min. Concentration of OBZ was determined by an external standard calibration curve. The HPLC eluent was introduced into the mass spectrometer using a pneumatically assisted electro-spray source (positive mode). The interface was adjusted to the following conditions: capillary voltage, 63 V; needle voltage, 4500 V; drying gas temperature, 300 °C. MS/MS spectra were obtained by collision-induced dissociation using nitrogen as the collision gas.

2.6 Determination of *O*-GT activity

O-GT activity was determined using the method described by San Miguel et al. [26]. The reaction mixture contained 0.1 mM substrate (quercetin, kaempferol), 2 mM uridine 5´-diphophoglucose disodium salt, 3.125 mM 4-nitrophenyl ß-D-glucuronide and 3.125 mM

salicin in 200 mM Tris/HCl buffer (pH 7.3) with 2 mM $MgCl_2$. The reaction was started by adding 100 µL enzyme extract. After 30 min incubation at 30°C the reaction was stopped by protein-precipitation with 10 µL concentrated phosphoric acid, after centrifugation at 15,000 × g for 2 min. The supernatant was diluted 1:4 (v/v) with HPLC solvent A. Measurement was performed by the HPLC system (Varian Pro-Star M215) equipped with a C18 Prontosil Spheribond column (5 µM, 250 × 3.0mm, Bischoff Chromatography, Leonberg). Mobile phases consists of 0.1% aqueous trifluoroacetic acid (TFA) as solvent A and acetonitrile with 0.1% TFA as solvent B. OBZ was separated with the following gradient: 0-8 min 92% B (isocratic); 8-9.5 min 100% B (linear increasing); 9.5-12.5 min 8% B (linear decreasing); 12.5-15 min 8% B(isocratic). The flow rate was kept at 0.85 mL/min and the analytes were detected via HPLC with UV detection at 370 nm (Varian ProStar 335, Germany). *O*-GT activity is expressed as the enzymatic formation of one pmole product per min [pkatal] in the enzyme extracts. Protein content was determined by the method of Bradford [27] with bovine serum albumin as a standard protein.

2.7 Data analysis

Statistical analyses were performed with the software GraphPad Prism v5.0. One-way analysis of variance (ANOVA) with Tukey post-test and two-way ANOVA with Bonferroni post-test were applied respectively to determine the significant differences between groups from seed germination tests and hairy roots experiment. Comparisons were considered significantly different for $p < 0.05$.

3 Results and discussion

3.1 Effect of OBZ and TiO_2 on plant growth

The potential effect of OBZ and TiO_2 on seed development was tested by calculating the germination rate and root length of tomato and barley. As shown in Fig. 1, treatment with OBZ and TiO_2 resulted in different germination patterns between tomato and barley. Addition of OBZ significantly inhibited germination rate of tomato seeds by 31.6%, accordingly, and the average root length of seeds germinated under OBZ treatment was 1.1 cm, which was 57.5% lower than that in the other treatments. Unlike studies which proposed the negative influence of nanoparticles on root length of tomato [28], this study did not detect effects of TiO_2 on seeds compared with non-treated seeds. However, TiO_2 reduced the impact of OBZ under the condition of simultaneous exposure, because the germination rate in the co-exposure was elevated and was similar to the control level,

moreover, slightly longer root length (nonsignificant) was observed for seeds exposed to treatments containing TiO_2. Similar to the present finding, Larue and co-workers reported that TiO_2 of concentrations up to 100 mg/L did not pose impact on wheat and rapeseed germination rates, and induction of root elongation was found for both plant species upon exposure [29]. Enhanced germination rate and growth was revealed in tomato grown in multi-wall nano-tube amended agar medium, presumably by affecting the expression of genes that facilitate cell division and development [30]. In agreement with the statement of Begum et al. the effects of nanoparticles differed among plant species [28]. The test with barley seeds in the present study shows no distinguished difference on the germination rate and root numbers (data not shown) among all the treatments, however, compared to the seedlings in the control, the root length was significantly reduced by 15.2 - 17.2% after addition of xenobiotics regardless of single or co-exposure treatment. The literature on the phytotoxicity of TiO_2 varies considerably with concentrations and plant species, Song et al. showed that high concentrations of TiO_2 inhibited the growth of *Lemna minor*, on the other hand, frond numbers were stimulated at low concentrations [31].

The favorable effect of TiO_2 was again confirmed by a microbiotest with *Lemna* in the present study. Regardless of single or co-exposure to TiO_2, the relative growth rate of fronds in these treatments was 47 - 49% significantly higher in comparison to the controls (Fig. 2). The results obtained from pigment measurements of corresponding fronds further support the aforementioned findings. Contents of chlorophyll (Chl) a and b were significantly lower in the plants exposed to OBZ compared with the controls, whereas addition of TiO_2 significantly increased Chl a, Chl b and carotenoids content by 42.2%, 63.8% and 46.5%, respectively, relative to the OBZ treatment alone (Fig. 3). Chlorophyll contents are important parameters to evaluate stress and toxicity to plants. Many studies have reported that TiO_2 could affect photosynthesis of plants, including variations on chlorophyll content, photosynthetic rate and chloroplast structure. For example, the chlorophyll content was increased in *Lemna* or mung bean subjected to TiO_2 [31,32], and it was explained that TiO_2 might enter the chloroplast and its oxidation-reduction reactions might accelerate electron transport and oxygen evolution [31]. TiO_2 has also been shown to stabilize the integrality of chloroplast membrane and protect the chloroplasts from aging [33]. Furthermore, net photosynthetic rate and Rubisco carboxylation was shown to be promoted under the treatment of TiO_2, as TiO_2 results in the enhancement of activity of Rubisco through the increase of mRNA amounts and protein expression [34]. Although oxidative stress has been observed in plants exposed to TiO_2 [35], the better growth of

plants under exposure to TiO_2 demonstrated that a certain amount of stress, which does not exceed the plant´s antioxidative capacity, might be positive for plants. This is supported by the opinion of Mittler that low level of reactive oxygen species (ROS) are beneficial, and are necessary for the activation of cellular proliferation, physiological function, and viability [36]. It was further speculated that in the case of dissociation of TiO_2, Ti^{4+} might transform the ROS O_2^- radicals which are damaging to the photosystem and may accelerate the aging [33].

3.2 Uptake of OBZ under co-exposure with TiO_2

During one week exposure to environmentally relevant concentrations of OBZ and TiO_2, OBZ was taken up continuously by roots under single exposure with OBZ and co-exposure with both compounds. As shown in Fig. 4, at the end of the experiment, uptake percentage reached 61.2% and 77.7% for OBZ alone and TiO_2 co-exposure, respectively. Roots from controls and TiO_2 treatment alone were excluded from the figure as no OBZ was detected in those samples. Co-exposure to TiO_2 significantly ($p<0.05$) increased accumulation of OBZ in roots. Compared to OBZ single exposure, the presence of TiO_2 enhanced OBZ amount in roots by 38% and 27% after 2 and 7 days incubation, respectively.

The majority of former studies had focused on the photo-degradation of organic pollutants in the presence of TiO_2 as catalyst [37,38], while OBZ is not supposed to be photodegradable due to its property as sun-blocker [39]. Its photo dynamics was proven to be stable in the co-existence of inorganic scatterer TiO_2, and very little interaction (adsorption) between the two species has been mentioned [40]. The results from medium free of roots confirmed the former findings, as shown in Fig. 5, concentration of OBZ remained consistent except on day-7 that 27.6% of OBZ was lost under the condition of co-exposure. Few studies have been carried out to investigate the role of TiO_2 on the accumulation of organic pollutants by plants. Uptake of TiO_2 alone has been reported in several plant species, and TiO_2 particles with smaller diameters accumulated to a greater extent than the larger ones [41]. In fact, plants may be able to increase the availability of TiO_2 by influencing the size of particles with root exudates, rhizosphere pH, etc. [17]. Nanoparticles of smaller size obtain new properties such as higher surface reactivity which might enlarge root pores or create new ones, leading to higher hydro-mineral flow and elevated nutrient uptake in root [29], a process that might simultaneously increase the uptake of OBZ and explain the higher OBZ concentration observed during co-exposure with TiO_2. The induced transport of secondary contaminants could also be attributed to the

physical damage caused by the nanomaterial. In recent studies, interactions between phenanthrene and nanomaterial have been visualized, and multiwall carbon nanotubes have been shown to pierce wheat root cell walls and by that enhance the uptake of phenanthrene into the living cells [42].

3.3 Effect of TiO_2 on transformation of OBZ

After uptake, OBZ underwent metabolic transformation. Possible pathways of OBZ in HRs have been described previously [25], and current results confirmed the hypothesis that part of the accumulated OBZ would be transformed to yield an oxybenzone-glucoside (OBZ-Glu) and subsequently an oxybenzone-malonyl-glucoside (OBZ-Mal-Glu). Effects of TiO_2 on the transformation of OBZ were not recognized until the 4^{th} day, when co-exposure with TiO_2 reduced the amount of OBZ-Glu to 76.5% of that in the OBZ alone condition (Fig. 6). The secondary transformation to OBZ-Mal-Glu was affected slightly by the addition of TiO_2, and a lower amount of OBZ-Mal-Glu was observed in the samples from co-exposure, while the difference was not statistically significant.

To further investigate the impact of TiO_2 on the detoxification mechanisms for OBZ, activity of *O*-glucosyltransferases (*O*-GT, E.C. 2.4.1.x) was measured as they are representative enzymes which participate in the phase II transformation of xenobiotics [43]. The involvement of the *O*-GT enzyme system is important in the responses of plant tissues to OBZ treatment, which have been confirmed formerly to catalyze the glucosylation of OBZ to form OBZ-Glu [25]. Activity of *O*-GT was evaluated with two natural substrates - quercetin and kaempferol. Consistent with previous findings showing that activities of *O*-GT were elevated to better transform the organic xenobiotic into less toxic form [44,45], the present study showed that after 4-day exposure in OBZ containing suspensions, *O*-$GT_{Quercetin}$ activities were stimulated significantly to 164.6% and 127.4%, and *O*-$GT_{Kaempferol}$ activities were enhanced to 131.2% and 118.3% of control for OBZ alone and co-exposure treatment, respectively (Fig. 7). Whereas co-exposure to TiO_2 showed 37.2% lower *O*-GT activity toward quercetin when compared to that level in the OBZ alone treatment. A similar pattern was also recorded for kaempferol as a substrate, where addition of TiO_2 to the OBZ treatment again lowered the *O*-GT activity, albeit the reduction was not statistically significant. The decrease of *O*-GT activity in enzyme extracts derived from the co-exposure experiments corresponds to the lower amount of OBZ-Glu detected in the roots from the same treatment. This result implies that TiO_2 affected the transformation of OBZ in plant. Inhibition of detoxification enzyme activities

in the co-exposure to TiO_2 has also been reported by Liu and co-workers. They report that the addition of TiO_2 to *Arabidopsis thaliana* exposed to tetracycline has reduced the activities of peroxidase and catalase [46]. In the present case, the inhibition effect was exaggerated when the hairy roots were treated with TiO_2 independently; the corresponding *O*-GT activity was suppressed significantly to 38 - 69% of the activity in non-treated hairy root tissue. After 7-day incubation, this negative effect was eliminated and the activity was recovered to the same level as that in controls. Similar inhibitory effects on the ability of plants to conjugate organic xenobiotics have also been found in plants subjected to heavy metals [47], and the same phenomenon has been demonstrated *in vitro* with isolated detoxification enzymes, which showed direct inhibition effect of cadmium on the catalytic reaction of glutathione reductase and glutathione-S-transferases [48].

4 Conclusion

The findings in the present study show that addition of TiO_2 may reduce the negative impact of OBZ, as higher germination rate and root length were observed in the co-exposure situation. Moreover, the highest growth rate and pigment contents were detected in *Lemna* grown under mixed pollution. Paradoxically, plants used for remediating OBZ can eventually be expected to encounter lower toxicity, since contaminants in mixture are more prevalent in the real environment. The hairy root system is an efficient model to study plant short term response with respect to OBZ and TiO_2. The results show that OBZ loaded with TiO_2 may increase the bioaccumulation of OBZ in plant, because more OBZ was incorporated into roots with the presence of TiO_2 in the hydroponic system. Activity of *O*-GT was elevated to detoxify the xenobiotic OBZ, and OBZ was continuously transformed to OBZ-malonyl-glucoside.

The present research complements the former results about the removal of single contaminant OBZ, as it aids in understanding the influence of nanoparticles on the plant remediation process of OBZ. The potential of phytoremediation must be scrutinized by considering detailed observations with mixed pollution, to better forecast the performance of plants under the influence of real world scenarios.

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Figures:

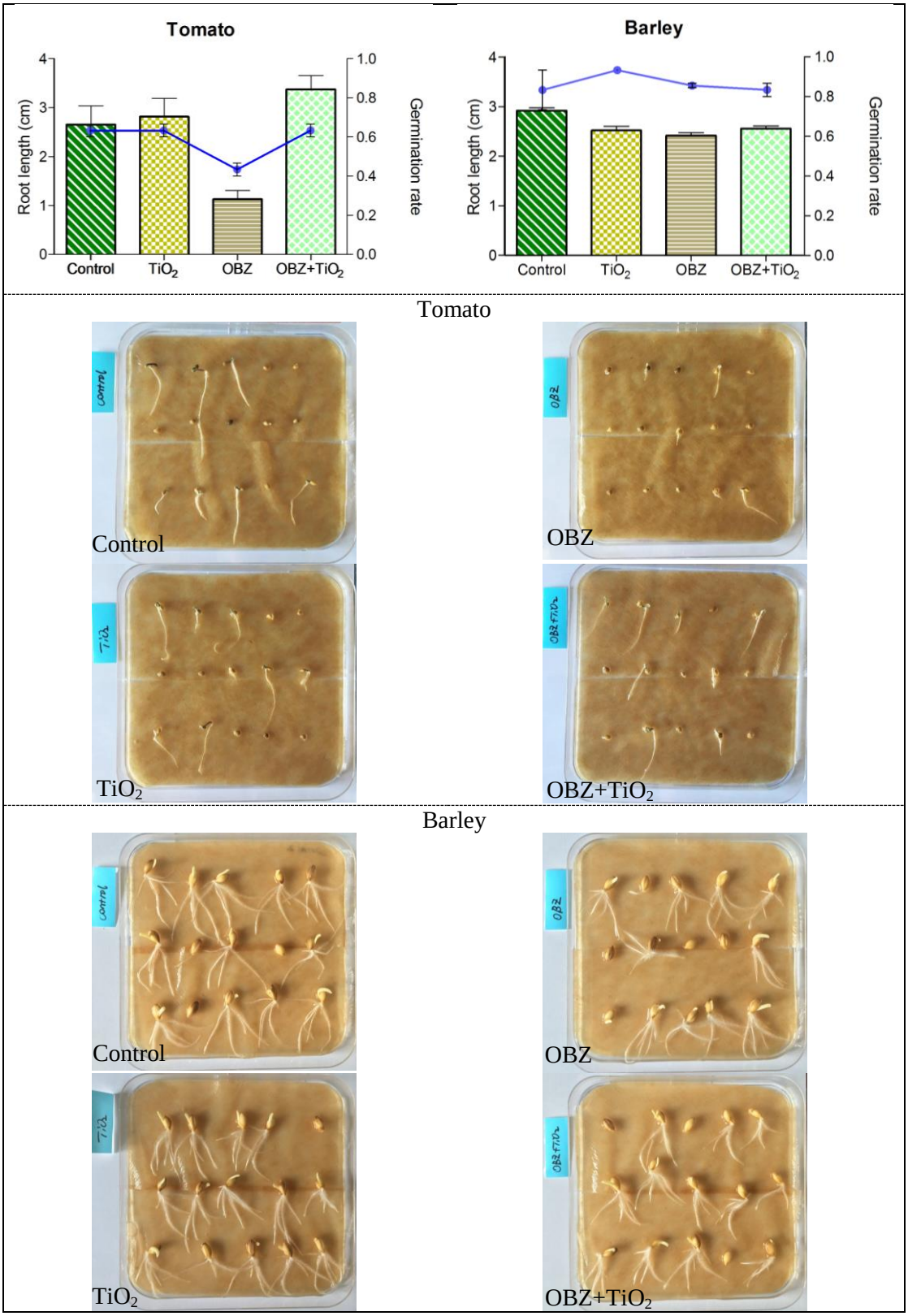
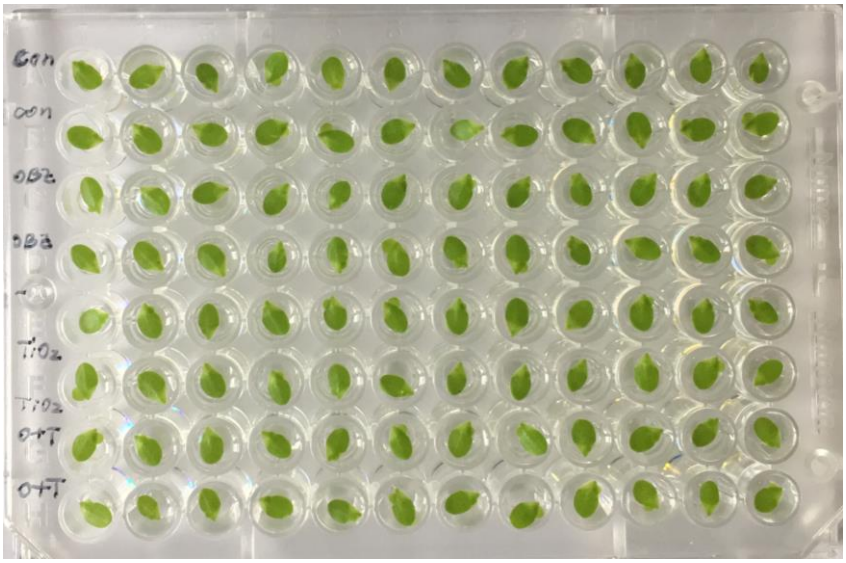
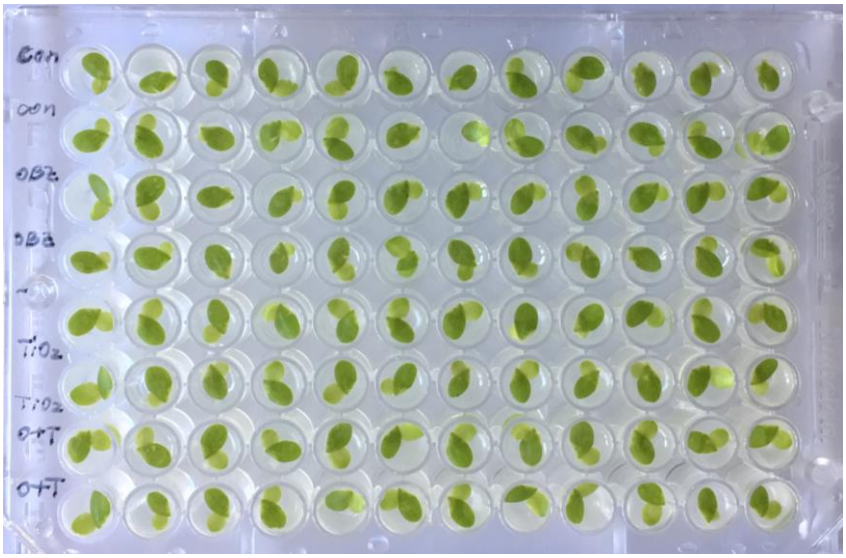


Figure 1 Effects of 3 mg/L TiO_2 and 5 µM OBZ on the germination rate (line) and root length (column) of barley and tomato seedlings. Image of seeds exposed to different treatments. Seeds were germinated on wet filter paper for 3 and 10 days for barley and tomato, respectively. Error bars represent standard error of mean (n=30).



0 d



3 d

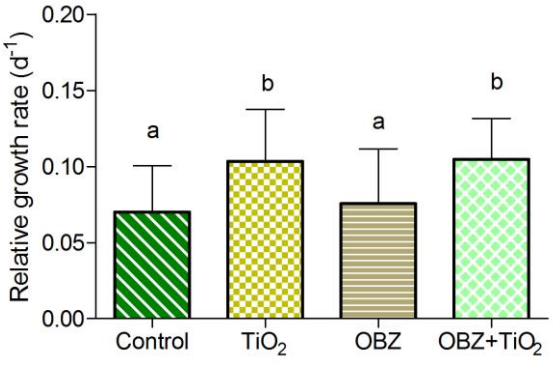


Figure 2 Impact of 3 mg/L TiO_2 and 5 μM OBZ on the growth of *Lemna minor*. The relative growth rate (RGR) was quantified by measuring the frond area of *Lemna* before and after incubation for 3 days. RGR = $(\ln A_3 - \ln A_0)/3$, A_0 is the initial frond area (cm^2) at day 0, A_3 is the area of corresponding frond at day 3. Error bars represent standard error of mean (n=24). Lowercase letters indicate significant differences among different treatment groups according to ANOVA at $p < 0.05$.

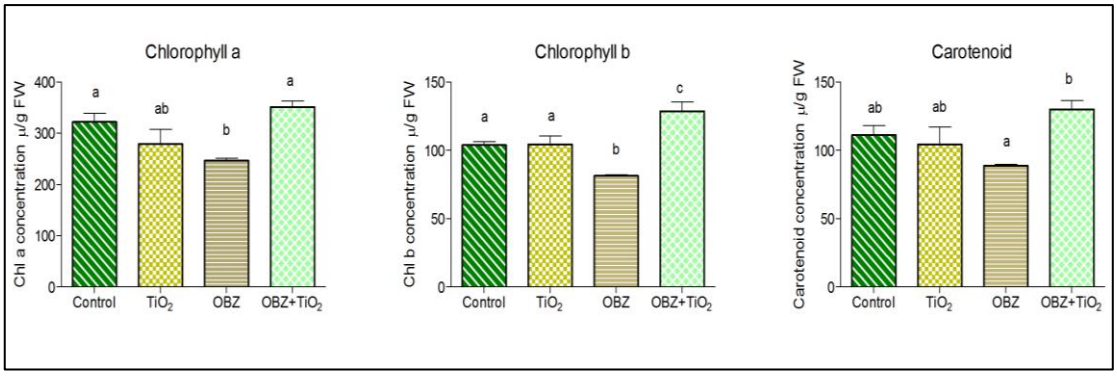


Figure 3 Contents of photosynthetic pigments after 3-day exposure to different treatments. Values are mean of two replicates, each replicate contained fronds from 12 wells. Error bars indicate SD. Lowercase letters indicate significant differences among different treatment groups according to ANOVA at $p < 0.05$.

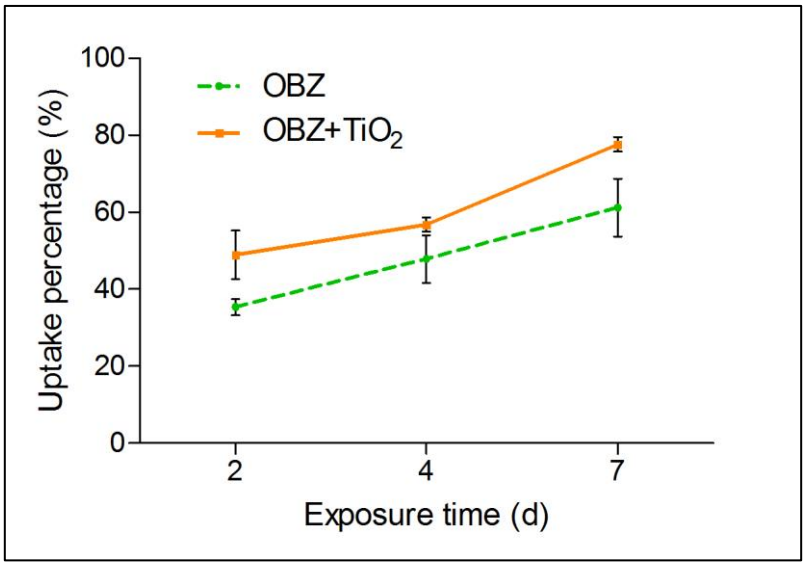


Figure 4 Effect of TiO_2 on the uptake of OBZ into horseradish hairy roots, the uptake percentage of OBZ (%) = the mass of OBZ taken up by roots under exposure (μg)/the initial mass of OBZ in the hydroponic medium (μg), the mass of OBZ in roots= roots weight (g)×OBZ concentration in root (μg g^{-1} fresh weight (FW)) Error bars indicate SD (n=3).

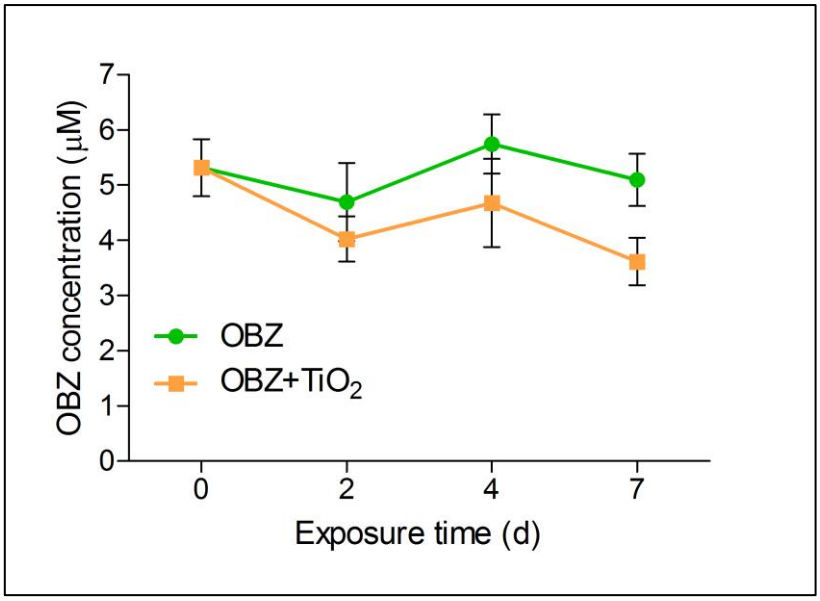


Figure 5 OBZ concentration (μM) in medium without roots over incubation time. No statistical difference among exposure time (except day 7) according to ANOVA at $p < 0.05$. Error bars indicate standard deviation (n=3).

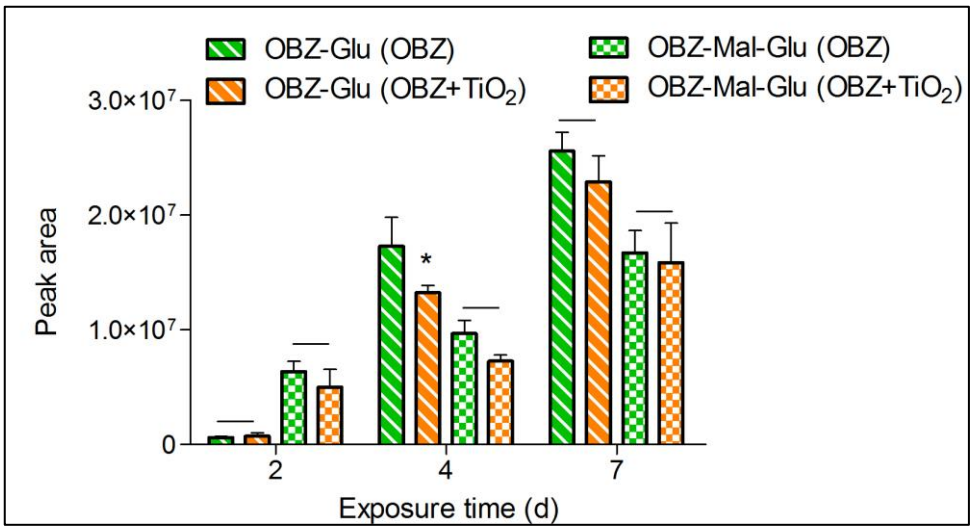


Figure 6 Impact of TiO_2 on the formation of OBZ metabolites in horseradish hairy roots from OBZ single treatment (OBZ-Glu (OBZ), OBZ-Mal-Glu (OBZ)) and co-exposure treatment (OBZ-Glu (OBZ+TiO_2), OBZ-Mal-Glu (OBZ+ TiO_2)) after 2, 4 and 7 days incubation. Values are mean of three parallel individuals, * p<0.05.

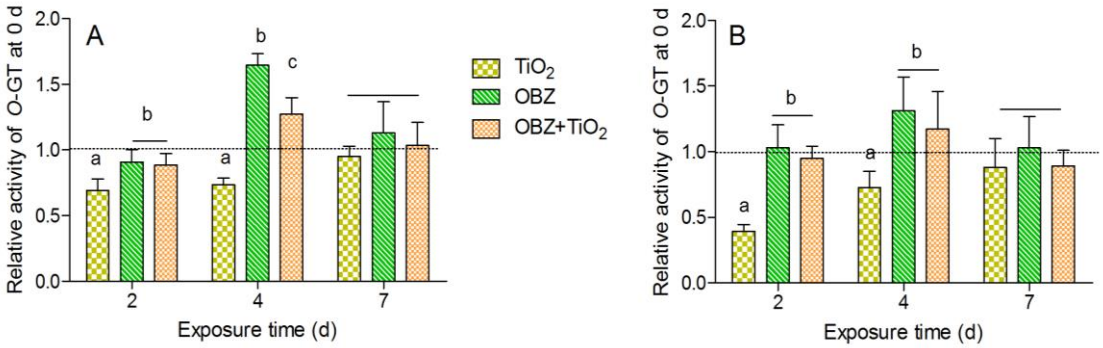


Figure 7 Influences OBZ and TiO_2 on the activities of *O*-GT in horseradish hairy roots collected from different treatments at each exposure time. *O*-GT activities (A: *O*-$GT_{Quercetin}$; B: *O*-$GT_{Kaempferol}$) are depicted relative to activities of root samples collected on the 0 d. Dashed lines are relative activities of control samples without of OBZ and TiO_2. Error bars indicate SD (n=3). Lowercase letters indicate significant differences among treatments according to ANOVA at $p < 0.05$.

Appendix B

Curriculum Vitae

Curriculum Vitae

Personal information

Feiran Chen
Born: 02.10.1988, Female
Nationality: PR China

Education

11.2013-present	PhD candidate Helmholtz Zentrum München, Munich, Germany
10.2011-10.2013	International Master of Environmental Science University of Cologne, Cologne, Germany
09.2007-07.2011	Bachelor of Forestry Nanjing Forestry University, Nanjing, China

Publications

Chen F, Huber C, May R, Schröder P. Metabolism of Oxybenzone in a hairy root culture: perspectives for phytoremediation of a widely used sunscreen agent, Journal of Hazardous Materials (2016), 230-236.

Chen F, Huber C, Schröder P. Fate of the sunscreen compound Oxybenzone in *Cyperus alternifolius* based hydroponic culture: uptake, biotransformation and phytotoxicity, Chemosphere (2017), 82:638-646.

Chen F, Schnick S, Schröder P. Concentration effects of the UV filter Oxybenzone in *Cyperus alternifolius*: assessment of tolerance by stress related response (under review with Environmental Science and Pollution Research).

He Y, Langenhoff A, Sutton N, Rijnaarts H, Blokland M, **Chen F**, Huber C, Schröder P. Metabolism of ibuprofen by *Phragmites australis*: uptake and phytodegradation. Environmental Science and Technology (2017), 51 (8), 4576–4584.

Conference contributions

10.2016	13th International Phytotechnologies Conference, Hangzhou, China Oral presentation, third place award
09.2015	15th EuCheMS International Conference on Chemistry and the Environment, Leipzig, Germany Poster
08.2012	Global Risk Forum, 4th International Disaster & Risk Conference, Davos, Switzerland Volunteer